Transcriptional Regulation of Intestinally and Hepatically Expressed Membrane Transporter Genes

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1 Summary

In humans, chemical solutes are selectively transported from the intestinal lumen into polarized intestinal epithelial cells. This intestinal absorption of drugs, nutrients and bile acids is mediated by uptake transporter proteins expressed in the apical enterocyte membranes. Expression levels of the intestinal transporters contribute to the degree of absorption of their substrates. They are important determinants for pharmacokinetics of many drugs and are frequently altered in diseases of the intestine, such as inflammatory bowel disease. Changes in transporter expression mediated by transcription factors may contribute to pathological processes or lead to aberrant drug absorption in affected patients.

The peptide transporter 1 (PEPT1) mediates uptake of di-/tri-peptides and peptidomimetic drugs in the small intestine. In patients suffering from chronic inflammation of the intestine, PEPT1 is aberrantly expressed in the colon. The transcriptional regulatory mechanisms leading to this dysregulation is unknown. A transcription factor which plays a pivotal role in chronic intestinal inflammation etiology is the peroxisome proliferator-activated receptor gamma (PPARγ). PPARγ binds as a heterodimer together with its heterodimerization partner retinoid X receptor α (RXRα) to palindromic DNA sequences within a target promoter and regulates the target gene expression. As the ability of PPARγ to regulate the human/rodent *PEPT1*/*Pept1* genes remains unelucidated, promoter activities of the human and rodent *PEPT1*/*Pept1* genes were comparatively studied in the intestine-derived cell line Caco-2.

In silico analysis revealed several putative PPARγ binding sites within the human and rodent promoters. Several human and rodent *PEPT1*/*Pept1* promoter constructs covering the putative PPARγ binding sites were cloned into pGL3Basic vectors containing luciferase genes to detect them by luminescence. Cells were co-transfected with the luciferase-linked *PEPT1*/*Pept1* promoter constructs and the expression plasmids encoding the PPARγ and RXRα genes. The reporter activities were measured by dual luciferase assays. PPARγ and RXRα proteins were translated *in vitro* and incubated with double-stranded oligonucleotides containing putative

PPARγ:RXRα binding sites of the *PEPT1/Pept1* promoter sequences. Complexes formed in these electrophoretic mobility shift assays (EMSAs) indicated a specific DNA binding by PPARγ:RXRα heterodimers.

In the presence of their respective ligands 15-deoxy-Δ12,14-prostaglandin J2 and 9-cis retinoic acid, the human and rat *PEPT1/Pept1* promoters were activated by PPARγ and RXRα. In contrast, the mouse *Pept1* promoter failed to respond. These results were shown in co-transfection assays, EMSAs employing *in vitro* translated proteins, Caco-2 cells transfected with expression plasmids for PPARγ and RXRα, and in rat ileal explants treated with ligands of PPARγ and RXRα. Despite these obtained results further analysis of the human and rat *PEPT1/Pept1* promoter with promoter constructs containing mutated PPARγ:RXRα DNA consensus regions to prevent the binding of the heterodimers and treatment of Caco-2 cells with specific ligands for PPARγ and RXRα to increase the PEPT1 mRNA did not change the activities of the promoters or the PEPT1 mRNA levels. It can be concluded that the human and rat *PEPT1/Pept1* promoters are both regulated by PPARγ and its heterodimerization partner RXRα. Whether this transcriptional regulation has an effect on the protein expression and transport activity of human and rat PEPT1/Pept1, needs further investigations.

Unlike PEPT1/Pept1, the organic anion transporting polypetides (OATPs/Oatps) are influx transporters with a broad spectrum of amphipathic substrates. They are widely expressed in human and rodent tissues. Due to their broad substrate specificity and tissue distribution they play a pivotal role in the pharmacokinetics of endogenous or exogenous compounds. It is known that the expression of some OATP/Oatp family members is influenced by vitamin D_3, the hepatocyte nuclear factor 1 α, the hepatocyte nuclear factor 3 β, the pregnane X receptor, the farnesoid X receptor and by chronic inflammatory conditions of the human intestine.

To understand the molecular mechanism that governs the transcriptional regulation of OATPs/Oatps, *in silico* analysis of the mouse *Slco1a1* and *Slco1a4* promoters were performed and several potential binding sites of pregnane X receptor (Pxr) and the glucocorticoid receptor (GR) were found. In further experiments, liver explants from two mice strains were incubated in media

containing agonists of Pxr (pregnenolone-16α-carbonitrile, dexamethasone, hyperforin), GR (dexamethasone) and an antagonist of GR (mifepristone). There was a clear tendency that the addition of pregnenolone-16α-carbonitrile and dexamethasone led to higher mRNA levels of Oatp1a1 and Oatp1a4 expressed in specimens of both strains. The addition of mifepristone and hyperforin did not result in a lower or higher Oatp1a1 and Oatp1a4 mRNA concentration. To further elucidate the transcriptional mechanisms that led to increased mRNA expression of the *Slco1a1* and *Slco1a4* genes, different promoter constructs of the *Slco1a1* and *Slco1a4* genes covering consensus regions of Pxr and GR were cloned into luciferase-linked pGL3Basic vectors. In co-transfection assays, the m*Slco1a1* promoter construct was activated by the mouse GR (mGr) and the mouse Pxr (mPxr) together with its heterodimerization partner mouse Rxrα in the human liver derived cell line HuH-7. The co-transfection of the mouse small heterodimerization partner (Shp), a repressor of other transcription factors activity, led to an increase of m*Slco1a1* promoter activity in the mouse liver derived cell line TIB-75. The mouse *Slco1a4* promoter was not activated in human and mouse liver derived cell lines by the human and mouse GR/Gr, PXR/Pxr and the human FXR or mouse Shp. Further molecular investigations are needed to completely understand the regulatory pathways controlling the m*Slco1a1* promoter activation by mouse Gr, Pxr:Rxrα and Shp. The m*Slco1a4* promoter activity needs a deeper analysis of potential transcription factors controlling the expression of the m*Slco1a4* gene.

2 Zusammenfassung

Nährstoffe, Arzneimittel oder körpereigene chemische Verbindungen wie z.B. Gallensalze werden aus dem menschlichen Darmlumen in Epithelzellen aufgenommen und von dort in den Blutkreislauf weitergegeben. Dies ist ein hochselektiver Prozess, welcher von Transportern, die in der Zellwand der Epithelzellen lokalisiert sind, gesteuert wird. Die aufgenommene Substratmenge und die Pharmakokinetik der einzelnen Substrate hängt somit stark von der Expression der entsprechenden Transporter in den Epithelzellen ab. Es ist bekannt, dass die

Expression durch den Gesundheitszustand des Darms reguliert wird. Bei entzündlichen Prozessen im Darm, wie z.B. chronisch entzündlichen Darmerkrankungen (Colitis ulcerosa, Morbus Crohn), kann die Expression der Transporter verändert sein. Dabei spielen Transkriptionsfaktoren eine wichtige Rolle. Durch sie kann die Genexpression der Transporter verändert und sowohl das Krankheitsbild beeinflusst werden wie auch zu einer veränderten Absorption von Medikamenten führen.

Der Peptidtransporter 1 (PEPT1) transportiert normalerweise Di- und Tripeptide oder Peptidomimetika. PEPT1 wird beim gesunden Mensch im Dünndarm exprimiert. Bei Patienten mit einer chronischen Entzündung des Darms wird dieser Transporter zusätzlich im Dickdarm exprimiert. Die Mechanismen, welche zu dieser transkriptionellen Dysregulation führen, sind bisher unbekannt. Ein Transkriptionsfaktor, welcher eine entscheidende Rolle bei chronischen Entzündungen des Darms spielt, ist der sog. ‚peroxisome proliferator-activated receptor γ' (PPARγ). PPARγ bindet als ein heterodimerer Proteinkomplex zusammen mit dem ‚retinoid X receptor α' (RXRα) an palindromische Sequenzen der DNA. Diese Sequenzen befinden sich unter anderem in der Promoterregion von Genen, welche Transportproteine kodieren. Durch die Bindung des Proteinkomplexes kann die Expression eines solchen Genes erhöht oder erniedrigt werden. Es ist bisher nicht bekannt, ob PPARγ die Expression des humanen *PEPT1* oder des *Pept1* Gens von Nagern beeinflussen kann. Aus diesem Grund wurde in der vorliegenden Studie untersucht, ob PPARγ an die Promoterregion des humanen *PEPT1* oder des *Pept1* Gens von Nagern bindet und die Genexpression beeinflussen kann. Die Studien wurden in humanen Caco-2 Zellen durchgeführt. In silico Untersuchungen zeigten, dass mehrere potentielle Bindungsstellen für PPARγ im humanen wie auch in Nagetier (Maus, Ratte) *PEPT1*/*Pept1* Promotoren vorhanden sind. Es wurden Promoterkonstrukte mit verschiedenen Bindungsstellen in den pGL3Basic Vektor kloniert, welcher ein Luziferase-Gen zur Lumineszenzanalyse exprimiert. Im pGL3Basic Vektor war die Expression des Luziferase-Gens unter direkter Kontrolle der Promoterkonstrukte, welche in den Vektor kloniert werden. Caco-2 Zellen wurden mit den humanen *PEPT1* und den *Pept1* Promoterkonstrukten der Maus und Ratte zusammen mit den Expressionsplasmiden für PPARγ und RXRα transfiziert,

um die Promoteraktivität der pGL3Basic Vektoren zu bestimmen. Beide Proteine, PPARγ und RXRα, wurden in vitro translatiert und mit zweisträngigen Oligonukleotiden, welche die möglichen Bindungsstellen des *PEPT1/Pept1* Promoters für PPARγ:RXRα Heterodimere enthielten, inkubiert. Die eingegangenen Komplexe wurden mittels ^{32}P-Markierung sichtbar gemacht und anhand ihrer Grösse mittels Gelelektrophorese aufgetrennt (EMSA). Diese Methode zeigte, dass eine spezifische Bindung der Oligonukleotide durch in vitro translatierte PPARγ:RXRα Heterodimere stattfand.

Es konnte gezeigt werden, dass der humane *PEPT1* und der *Pept1* Promoter der Ratte durch PPARγ:RXRα Heterodimere aktiviert wurde. Im Gegensatz dazu wurde der *Pept1* Promoter der Maus nicht aktiviert. Dieses konnte in verschiedenen Versuchen gezeigt werden, wie: Co-Transfektionen, EMSA-Versuchen mit in vitro translatierten Proteinen, Caco-2 Zellen transfiziert mit Expressionsplasmiden für PPARγ und RXRα und behandelt mit deren Agonisten, Proben aus dem Ileum von Ratten behandelt mit PPARγ und RXRα Liganden. In Experimenten, bei denen die Bindungsstellen im humanen oder im Promoter der Ratte mutiert waren, um eine Bindung von der PPARγ:RXRα Heterodimere zu verhindern, wurden die Promotoren trotzdem aktiviert. Die Zugabe von PPARγ und RXRα Agonisten in das Zellmedium von Caco-2 Zellen führte zu keiner Erhöhung der PEPT1 mRNA. Zusammenfassend lässt sich sagen, dass die Aktivität des humanen und des Promoters der Ratte durch die Bindung von PPARγ:RXRα Heterodimeren gesteigert wurde. Ob dies eine Auswirkung auf die Proteinexpression oder die Transportaktivität von PEPT1 und rPept1 Proteinen hat, benötigt weitere Experimente.

Die ‚organic anion transporting polypetides' (OATP/Oatps) sind ebenfalls Aufnahmetransporter. Sie transportieren jedoch amphipathische statt peptidomimetische Substanzen. Im Mensch und in Nagern sind die OATP/Oatps in zahlreichen Zellen und Organen exprimiert. Wegen ihren zahlreichen Substrate und der hohen Exprimierung spielen sie eine wichtige Rolle in der Pharmakokinetik von endogenen oder exogenen Verbindungen. Bisher ist bekannt, dass die Expression der OATP/Oatps durch Vitamin D_3, den ‚hepatocyte nuclear factor α', den ‚hepatocyte nuclear factor 3 β', den ‚pregnane X receptor', den ‚farnesoid X receptor' und durch

chronische Entzündungen des Darms beeinflusst wird. Es sind jedoch nur wenige Kenntnisse über die molekularen Mechanismen, welche zu diesen transkriptionellen Veränderungen führen, vorhanden. Deshalb wurde eine in silico Analyse der *Slco1a1* und *Slco1a4* Gene der Maus durchgeführt, die zur Auffindung mehrerer Bindungsstellen für die beiden Transkriptionsfaktoren ‚pregnane X receptor' (Pxr) und ‚glucocorticoid receptor' (GR) führte.

Basierend auf diesen Erkenntnissen wurden Leberproben von 2 Mäusestämmen in Zellkulturmedien inkubiert, welche Agonisten für Pxr (Pregnenolon-16α-carbonitril, Dexamethason, Hyperforin) und GR (Dexamethason), sowie einen Antagonisten für GR (Mifepriston) enthielten. Danach wurde die Slco1a1 und Slco1a4 mRNA mit real-time PCR analysiert. In beiden Stämmen führte die Zugabe von Pregnenolon-16α-carbonitril und Dexamethason zu einer erhöhten Expression der Slco1a1 und Slco1a4 mRNA. Die Zugabe von Mifepriston und Hyperforin bewirkte jedoch keine Veränderungen der Slco1a1 und Slco1a4 mRNA. Verschiedene Promoterkonstrukte, welche mögliche Pxr und GR Bindungsstellen der *Slco1a1* und *Slco1a4* Promotoren enthielten, wurden in den pGL3Basic Vektor kloniert und die Aktivität der Promoterkonstrukte mit Chemolumineszenz analysiert. Es konnte gezeigt werde, dass in Co-Transfektionen der *Slco1a1* Promoter durch GR und durch Pxr zusammen mit seinem Heterodimerisationspartner Rxrα in der humanen Leberzelllinie HuH-7 aktiviert wurde. In Co-Transfektionen in einer Leberzelllinie von Mäusen, TIB-75, führte die Zugabe des ‚small heterodimerization partner' (mShp), eines Repressors von Transkriptionsfaktoren, zu einer erhöhten Aktivität des *Slco1a1* Promoters. In humanen oder Leberzelllinien der Maus zeigte der *Slco1a4* Promoter keine erhöhte Aktivität durch Zugabe von GR/mGr, PXR/mPxr, FXR oder mShp Expressionplasmiden zu Co-Transfektionen. Weitere Experimente sind nötig, um die Aktivierung des *Slco1a1* Promoters durch mGr, mPxr:mRxrα und mShp zu klären. Im Falle des *Slco1a4* Promoters müssen eventuell andere Transkriptionsfaktoren in Betracht gezogen werden, welche Einfluss auf die Transkription haben könnten.

3 List of abbreviations and ligands

9-cis-RA: 9-cis-retinoic acid (agonist of RXRα)

15d-PGJ2: 15-deoxy-Δ12,14-prostaglandin J2 (agonist of PPARγ)

γ-globulin: bovine γ-globulin

acrylamide/bis: acrylamide and bis-acrylamide solution

ACE: angiotensin converting enzyme

AHPN: 6-[3-(1-adamantyl)-4-hydroxyphenyl]-2-naphthalene carboxylic acid (agonist of mShp**)**

ASBT: apical sodium-dependent bile acid transporter

ATCC: American Type Culture Collection

ATG: adenosine thymidine guanosine (start of translation)

APS: ammonium peroxo disulfate

BCA: bicinchoninic acid

Carb: carbenicillin

CDCA: chenodeoxycholic acid (agonist of FXR)

CER I: cytoplasmic extraction reagent I

CER II: cytoplasmic extraction reagent II

ChIP: chromatin immunoprecipitation

Ciprofibrate (agonist of PPARα)

CO_2/RH: carbon dioxide/relative humidity

cpm: counts per minute

Ct: cycle threshold

DCA: deoxycholic acid

ddH_2O: double distilled water

Dexamethasone (agonist of GR and PXR)

dGTP/dCTP/dTTP: mixture of desoxyguanosine triphosphate/ desoxycytosine triphosphate/ desoxythymidine triphosphate

DMEM: Dulbecco's Modified Eagle Medium

DMSO: dimethyl sulfoxide

DPDPE: (2-$_D$-penicillamine, 5-$_D$-penicillamine)-enkephalin

DR motif: direct repeat motif

DTT: dithiothreitol

EGF: epidermal growth factor

EMSA: electromobility shift assay

ER: estrogen receptor

ER motif: everted repeat motif

FBS: fetal bovine serum

FXR: farnesoid X receptor

GR: glucocorticoid receptor

GW-9662 (antagonist of PPARγ)

GW-1929 (agonist of PPARγ)

HGNC: HUGO Gene Nomenclature Committee

HNF1α: hepatocyte nuclear factor 1 α

HNF3β: hepatocyte nuclear factor 3 β

HNF4α: hepatocyte nuclear factor 4 α

HRP: horse radish peroxidase

HUGO: Human Genome Organization

Hyperforin (agonist of PXR)

IBD: inflammatory bowel disease

IFNγ: interferon γ

IR: inverted repeat motif

LB: lysogeny broth

LCA: lithocholic acid

MDR: multidrug resistance protein

Mifepristone (antagonist of GR)

MK-886 (antagonist of PPARα)

Motif: specific DNA sequence bound by a transcription factor

MRP: multidrug resistance-related protein

MRP2: multidrug resistance-related protein 2

Na^+/K^+-ATPase: sodium/potassium adenosine-5'-triphosphatase

NER: nuclear extraction reagent

NTCP: sodium taurocholate co-transporting polypeptide

OATP: organic anion transporting polypeptide

PBS: phosphate buffered saline

PCN: pregnenolone-16α-carbonitrile

PCR: polymerase chain reaction

PEPT1: peptide transporter 1

PG: prostaglandin

PIC: protease inhibitor cocktail

PMSF: phenylmethane sulfonylfluoride

poly(dI-dC)-poly(dI-dC): poly(deoxyinosinic-deoxycytidylic)-poly(deoxyinosinic-deoxycytidylic) acid sodium salt

PPAR: peroxisome proliferator-activated receptor

PPARα: peroxisome proliferator-activated receptor α

PPARγ: peroxisome proliferator-activated receptor γ

PPRE: peroxisome proliferator-activated receptor response element

PXR: pregnane X receptor

RIPA: radioimmunoprecipitation assay

rcf: relative centrifugal force

RISC: RNA-induced silencing complex

rpm: revolutions per minute

RT: room temperature (~22 °C)

RXRα: retinoid X receptor α

SD: standard deviation

SHP: small heterodimer partner

siRNA: small interfering RNA

SLC: solute carrier

TBE buffer: Tris-Borate-EDTA buffer

TEMED: tetramethylethylenediamine

TM: transmembrane

TNFα: tumor necrosis factor α

Troglitazone (agonist of PPARγ)

Trizma: 2-Amino-2-(hydroxymethyl)-1,3-propanediol

VDR: vitamin D receptor

WY-14643 (agonist of PPARα)

4 Introduction

Lipid bilayers of cells separate the intracellular space from the extracellular environment and are essential for the functioning of living cells. Depending on the chemical structure of a compound, this bilayer can be an obstacle for molecules that is extremely difficult to cross. However, cells have to take up and excrete selectively or unselectively nutrients, waste products and signaling molecules from the extracellular into the intracellular space or vice versa. Most lipophilic compounds can cross biological membranes through diffusion, not so hydrophilic or charged compounds which poorly pass the lipid bilayer this way. They have to be transported across the lipid barrier with the support of integral proteins.

The simplest way of transport is diffusion which does not require integral proteins coupled to a biochemical energy source. If the transport requires energy to drive the influx or efflux via membrane proteins or simply needs proteins as a hydrophilic gate in the membrane, three types of transport mechanisms can be distinguished. First a membrane protein can form a pore which is always open, as shown for perforin. Originally hypothesized by Podack and co-workers (Podack et al., 1984), it is today known that perforin inserts itself into the target cell membrane forming a pore which allows granzymes to influx into the cell and induce apoptosis (Voskoboinik et al., 2006, Law et al., 2010). This pathway represents a transport mechanism which is called passive transport because it does not require an energy source for the functioning of the membrane protein since the flux of the solutes follows the concentration gradient.

A second transport mechanism requires transporters coupled to energy sources. By this way the transport of solutes is possible against the concentration gradient. Energy requiring transporters can again be divided into two different groups. First, carriers can be coupled to an electron transport chain or the hydrolysis of ATP (e.g. Na^+/K^+-ATPase). Each carrier protein has a specific affinity to one or several solutes and transports them across the membrane bilayer. Second, carriers which are not coupled to an electron transport chain or do not hydrolyze ATP are called solute carriers (SLCs). They receive their energy by coupling the uphill transport of a solute with the downhill transport of another solute. The SLC

superfamily is classified according to the homology of the amino acid sequences. Currently it includes 51 families and 378 transporter genes. The nomenclature system was proposed by the HUGO Gene Nomenclature Committee (HGNC) (Wain et al., 2002) and represents the names of the genes that encode the transporters. Members within a SLC family have at least 20-25% amino acid sequence homology to each other. Numerous *SLC* transporter gene defects have been identified and shown to lead to disease symptoms or to cause pathophysiological conditions in the metabolism thus supporting the urgent need for research in this field. For example, defects in the human apical sodium-dependent bile acid transporter ASBT result in malabsorption of bile acids in the ileum (Oelkers et al., 1997). The absence of MRP2 in human hepatocytes leads to the Dubin-Johnson syndrome, an autosomal recessive disorder causing conjugated hyperbilirubinemia (Wada et al., 1998). Another example is the cystic fibrosis that is characterized by an abnormal function or absence of the cystic fibrosis transmembrane conductance regulator (Riordan et al., 1989) severely affecting the lung, pancreas, intestine and liver of patients.

Organic anion transporting polypeptides (OATPs) are sodium independent influx transporters of the SLCO21 family with a broad spectrum for amphipathic substrates. Extensively studied in humans, they are expressed in the liver, blood-brain barrier, choroid plexus, lung, heart, intestine, kidney, placenta and testis (Tamai et al., 2000). Due to their broad substrate specificity they play a pivotal role in the pharmacokinetics of endogenous and exogenous compounds. Their substrates include bile salts (Kullak-Ublick et al., 1994, Eckhardt et al., 1999, Reichel et al., 1999), organic dyes (van Montfoort et al., 1999), thyroid hormones (Friesema et al., 1999) and steroid conjugates (Eckhardt et al., 1999, Reichel et al., 1999, Bossuyt et al., 1996, Kanai et al., 1996a, Kanai et al., 1996b) among others. Together with the multidrug resistance proteins (MDRs) and the multidrug resistance-related proteins (MRPs) they play an important role in the context of drug uptake and excretion.

The driving force of OATPs/Oatps to transport their substrates is not sodium dependent (Jacquemin et al., 1994, Noé et al., 1997, Walters et al., 2000) like ASBT or the sodium taurocholate co-transporting polypeptide NTCP. Shi and co-workers (Shi et al. 1995) found a bidirectional transport of sulfobromophthalein in rat Oatp1a1 expressing HeLa cells. Satlin and co-workers (Satlin et al., 1997) studied the

taurocholate/hydrogencarbonate exchange by rat Oatp1a1 in HeLa cells. Hydrogencarbonate can be suggested as the extruded anion, but other ions are also possible for the exchange *in vitro*. For instance glutathione has been shown to contribute to this uptake (Li et al., 1998).

In recent years, 36 OATPs/Oatps have been identified and classified in agreement with the HGNC recommendations in human, mice and rats. OATPs/Oatps within the same family share ≥40% amino acid sequence identities and are designated with numbers from 1 to 6 (OATP1 to OATP6). The subfamilies include OATPs/Oatps with amino acid sequence of ≥60% identities. Letters are used to label the different subfamilies (e.g. OATP1A, OATP1B, OATP1C, etc.). Individual proteins within the same subfamily are designated with numbers (e.g. OATP1A2, Oatp1a1, Oatp1a3, etc.). The OATP1 family consists of four subfamilies. The three subfamilies OATP1A/Oatp1a, OATP1B/Oatp1b and OATP1C/Oatp1c contain 28 individual genes in human, mice and rats. All OATPs/Oatps exhibit 12 transmembrane (TM) domains based on hydropathy analysis (Jacquemin et al., 1994). They have an extracellular domain between the TMs 9 and 10 and two *N*-glycosylation sites in the extracellular loop 2 and one in loop 5. Between the extracellular loop 3 and TM domain 6, a highly conserved consensus sequence was identified (Hagenbuch and Meier, 2003). This amino sequence is also known under the label 'OATP superfamily signature' and can be found in the mammalian species human, mouse and rat, but also in the phylogenetically rather distant *Caenorhabditis elegans* and *Drosophila melanogaster*.

The OATP1A subfamily contains one human member, OATP1A2 (gene symbol *SLCO1A2*). It was originally cloned from a human liver cDNA library (Kullak-Ublick et al., 1995). The authors demonstrated that OATP1A2 has its strongest expression in the blood-brain barrier. The liver and kidneys have a weaker expression. OATP1A2 has an apparent molecular mass of ~ 85 kDa (Kullak-Ublick et al., 1997), whereas in brain capillary endothelial cells its apparent molecular mass is ~ 60kDa (Gao et al., 2000). OATP1A2 transports bile salts, steroid conjugates, prostaglandin E2, the endothelin receptor antagonist BQ-123, the thrombin inhibitor CRC-200, the opioid receptor agonists (2-D-penicillamine, 5-D-penicillamine)-

enkephalin (DPDPE), deltorphin II, the antihistamine fexofenadine, the poisonous cardiac glycoside ouabain and the cyanobacterial toxin microcystin (Hagenbuch et al., 2003). OATP1A2 has four mouse [Oatp1a1 (Hagenbuch et al., 2000), Oatp1a4 (van Montfoort et al., 2002), Oatp1a5 (Walters et al., 2000), Oatp1a6 (Choudhuri et al., 2001)] and four rat homologues [Oatp1a1 (Jacquemin et al., 1994), Oatp1a3 (Saito et al., 1996), Oatp1a4 (Noé et al., 1997), Oatp1a5 (Abe et al., 1998)] within the OATP1A/Oatp1a subfamily.

The transcriptional regulation of several OATPs/Oatps has been studied in more detail. In Caco-2 cells treated with vitamin D_3, the endogenous expression of OATP1A2 mRNA was induced through the vitamin D_3 receptor at the transcriptional level (Eloranta et al., 2012). In hepatocyte-derived liver cell lines, Jung and co-workers showed that the hepatocyte nuclear factor 1α (HNF1α) binds to its preferred DNA consensus region in the *SLCO1B1* promoter and activates it (Jung et al., 2001). The transcriptional regulation of the *SLCO1B3* gene was dependent on HNF1α (Jung et al., 2001) and in addition on the farnesoid receptor X (FXR) (Jung et al., 2002).The hepatocyte nuclear factor 3β (HNF3β) represses the transcription of the *SLCO1B3* gene shown by decreased levels of OATP1B3 mRNA and protein in hepatocellular carcinoma samples from patients (Vavricka et al., 2004). A possible link between inflammatory signaling and transcriptional regulation of OATPs in the human intestine was shown by Wojtal and co-workers (Wojtal et al., 2009). In the ileum and colon of IBD patients the mRNA levels of OATP2B1 and OATP4A1 were upregulated. Guo and co-workers found that in the rat liver cell line H4IIE, pregnenolone-16α-carbonitrile (PCN), a ligand of the rodent pregnane X receptor (PXR), induces rat *Slco1a4* gene expression (Guo et al., 2002). Several PXR response elements were identified in the promoter region of rat the *Slco1a4* gene. The binding of PXR together with its heterodimerization partner retinoid X factor α (RXRα) to these PXR response elements was shown in electrophoretic mobility shift assays. Luciferase assays confirmed the induction of the rat *Slco1a4* promoter by the activation of PXR by PCN. Staudinger and co-workers showed that the bile acid lithocholic acid (LCA) activates PXR and regulates the expression of Oatp1a4 proteins in mice (Staudinger et al., 2001). The mRNA and protein expression of rat Oatp1a4 was also increased in rats treated with phenobarbital for 5 days (Hagenbuch

et al., 2001). Phenobarbital, a PXR activator, is also an important inducer of several biotransformation enzymes like cytochrome P-450 monooxygenase (Waxman et al., 1992) or gluthatione *S*-transferase (Pinkus et al., 1993).

So far, only Oatp1a/1b knockout mice have been bred by van de Steeg and co-workers (van de Steeg et al., 2012). But Oatp expression has been evaluated in several transcription factor knockout mice models. HNF1α knockout mice have decreased expression of Oatps in the liver (Shih et al., 2001). This results in impaired basolateral membrane bile acid uptake and augmented plasma bile acid concentrations. Mice with a conditional hepatocyte nuclear factor 4α (HNF4α) gene knockout in mature hepatocytes developed decreased expression levels of Oatp1a1 (Hayhurst et al., 2001). These studies led to the assumption that HNF1α and HNF4α are important factors regulating Oatp expression in mice. FXR null mice fed with cholate showed increased Oatp1a1 expression (Sinal et al., 2000). The molecular regulatory mechanism of this induced expression is unknown. Oatp1a4 mRNA expression was increased upon treatment with PCN in wild type mice but not in PXR null mice (Staudinger et al., 2001). In several models of cholestasis such as bile duct ligation or ethinyl estradiol treatment, Oatp protein expressions were downregulated (Meier et al., 2002).

Another family of uptake transporter is represented by the human ileal apical sodium dependent bile acid transporter ASBT (gene symbol *SLCO10A2*). ASBT belongs to the *SLC10* family of solute carriers. It is the main reabsorptive transporter of bile acids from the intestinal lumen. The extent of absorption influences the bile acid pool size and the activity of the bile acid synthesizing enzymes cholesterol-7α-hydroxylase and cholesterol-27-hydroxylase (Xu et al., 2000). The human ASBT protein was first cloned and detected by Craddock and co-workers in the terminal ileum, caecum and kidney (Craddock et al., 1998).

ASBT transports conjugated and unconjugated bile salts and is sodium ion dependent. The affinity for chenodeoxycholic acid (CDCA) and deoxycholic acid (DCA) is higher than for taurocholate (Craddock et al., 1998). Jung and co-workers have shown that the peroxisome-proliferator activated receptor α (PPARα), activated by ciprofibrate (an anti-hypercholesterolemia drug), or the synthetic ligand WY14643 binds to the so called direct hexanucleotide DNA motif (DR motif) of the *ASBT*

promoter and leads to an activation of the ASBT promoter in human colon derived Caco-2 cells. The nuclear receptor PPARα binds to the DNA motif as a heterodimer together with RXRα (Kliewer et al., 1992)

Duane and co-workers revealed that patients with type IV hypertriglyceridemia exhibit decreased intestinal bile salt absorption (Duane et al., 2000) and showed that the expression of ASBT mRNA and protein levels were significantly reduced in the intestine of patients. Fatty acids are known activators of PPARα which leads to the assumption of a link between lipid metabolism and bile salt absorption by ASBT. The glucocorticoid receptor (GR) is a well known transcriptional regulator of the *SLCO10A2*gene in humans. This nuclear steroid hormone receptor is activated by glucocorticoids such as dexamethasone and forms homodimers to bind the DNA motif. Jung and co-workers (Jung et al., 2004) showed the *in vitro* binding of two commonly used glucocorticoids, budenoside and dexamethasone, to two DNA response elements in the *ASBT* promoter region and found an activation of the promoter in Caco-2 and HuH-7 cells. Patients with Crohn's disease, an inflammatory condition of the intestine, often have decreased ASBT protein levels and consequently suffer from bile acid malabsorption. It could be shown that patients under budenoside medication showed higher ASBT protein levels in the small intestine compared to patients without budenoside treatment, thus supporting the influence of glucocorticoids on the transcriptional regulation of the *ASBT* gene.

A further family of uptake transporters transports mainly oligopeptides and is proton driven in contrast to the majority of the other uptake transporters which are sodium ion dependent. The human peptide transporter 1 (PEPT1), gene symbol *SLC15A1*, is located in the brush border membrane of the small intestine and to a lesser degree in proximal renal tubule cells (Fei et al., 1994). PEPT1 contains 12 transmembrane domains predicted by hydropathy analysis (Covitz et al., 1998). Although the function of this transporter is primarily the absorption of di- and tripeptides, PEPT1 transports a wide spectrum of peptidomimetic substrates. Compounds such as the antitumour agent bestatin (Saito et al., 1996), the angiotensin-converting enzyme (ACE) inhibitor captopril (Zhu et al., 2000), the β-lactam antibiotic cefadroxil (Boll et al., 1994) and the antiviral drug acyclovir (Han et

al., 1998) are substrates of PEPT1. The energy for the transport is generated by an inwardly directed proton flow (Biegel et al., 2006).

PEPT1 has recently become of interest because its expression is regulated by nutrition and is involved in the obesity problem. The small intestinal Pept1 mRNA expression level increases when rats were fed a diet supplemented with high amounts of protein (Erickson et al., 1995). Shiraga and co-workers showed with the same rat model an increase in Pept1 protein levels and augmented uptake of dipeptides when the rats were fed with a high protein diet (Shiraga et al., 1999). The authors suggest that most likely the increase in Pept1 protein level is the result of promoter activation by dipeptides or by the amino acids phenylalanine, arginine or lysine. It has also been argued that the increase could be the consequence of more stable Pept1 mRNA (Walker et al., 1998). Pan and co-workers showed that starvation of rats over four days significantly increases the transport of the PEPT1 substrate ceftibuten (Pan et al., 2003). Habold and co-researches confirmed by immunostaining and western blotting that the Pept1 protein expression in rat enterocytes increased after twelve days of starvation (Habold et al., 2007). Nässl and co-authors (Nässl, et al., 2011) hypothesized a cross-talk of arginine and leptin signaling in mouse brain, which led to reduced food intake of Pept1 knockout mice over up to five days compared to wildtype control animals when fed with a high protein diet (45 protein energy percent). After five days the knockout mice did not show an increase in body weight despite a regain in food intake. A reduction in energy assimilation and augmented fecal energy loss on a high-protein diet were discussed. Plasma arginine levels were elevated in the knockout mice on high protein and normal protein diet compared to the wildtype mice, but the plasma leptin levels were reduced only in mice fed with a high protein diet.

The PPARα synthetic ligand WY-14643 led to increased PEPT1/Pept1 mRNA expression when administered to Caco-2 cell layers and rats. Furthermore, the uptake of the dipeptide glycylsarcosine was augmented after treatment of Caco-2 cells with the ligand. Comparing PPARα knockout and wildtype mice during fasting, the increase of Pept1 mRNA was not observed in the knockout mice (Shimakura et al., 2006). This study supports a possible role of PPARα in fasting-induced Pept1 expression in rodents.

The expression of the human and rodent PEPT1/Pept1 genes is also affected by several hormonal pathways. Hyperthyroidism decreases the intestinal Pept1 expression in rats either through an interaction between the thyroid hormone and the rat *Pept1* promoter, or by increasing Pept1 mRNA stability (Ashida et al. 2002, Ashida et al., 2004). Leptin increased PEPT1/Pept1 protein levels and transport activity of cephalexine into Caco-2 cells and in the small intestine of rats (Buyse et al., 2001). Gangopadhyay and co-workers showed that diabetic rats had augmented Pept1 protein levels and transport activity in their brush border membrane and suggested a role of insulin in the transcriptional regulation (Gangopadhyay et al., 2002). Addition of epidermal growth factor (EGF) to the medium of the cell culture led to a decreased uptake of glycylsarcosine into Caco-2 cells (Nielsen et al., 2001). Not only hormones seem to regulate PEPT1/Pept1 expression, but also ions like Ca^{2+}, cytokines and drugs are involved in the regulation of the *PEPT1/Pept1* genes. Decreased intracellular Ca^{2+} levels augmented absorption of cefixime mediated by PEPT1 into Caco-2 cells when they were treated with Ca^{2+} channel blockers like nifedipine and verapamil (Wenzel et al., 2002). In chronic states of inflammation in the human intestine, PEPT1 is expressed in specimens of inflamed colon, whereas it is not expressed in the healthy colon (Merlin et al., 2001). Vavricka and co-workers studied the uptake of glycylsarcosine into Caco-2 cells and in mice injected with cytokines. The tumor necrosis factor α (TNFα) and interferon γ (IFNγ) both increased PEPT1/Pept1 transport activity in the membrane (Vavricka et al., 2006). Pentazocine, a synthetic opioid, raised the transport activity of PEPT1 in Caco-2 cells by increasing PEPT1 mRNA in a dose-dependent manner (Fujita et al., 1999).

Several examples have been found that the transcriptional regulation of genes encoding transporter proteins can lead to severe symptoms or cause diseases. Thus, the present thesis focuses on the better understanding of the molecular mechanisms governing transcriptional regulation of transporter genes encoding human and rodent OATPs/Oatps and PEPT1/Pept1 proteins.

5 Aims

- To study whether the transcription of the *SLC15A1* gene, encoding the protein PEPT1, is regulated by the peroxisome proliferator-activated receptor γ (PPARγ) and its heterdimerization partner retinoid X receptor α (RXRα). These studies include assays analyzing the *PEPT1* and the mouse and rat *Pept1* promoter *in silico* and *in vitro*. Additionally assays measuring a potential effect of treatment of Caco-2 cells or ileal rat explants with ligands for PPARγ and RXRα on the PEPT1/Pept1 mRNA expression in the cells are applied.

- To study whether the mouse *Slco1a1* and *Slco1a4* promoters coding for Oatp1a1 and Oatp1a4 are transcriptionally regulated by the glucocorticoid receptor (GR), the pregnane X receptor (PXR) and the farnesoid X receptor (FXR) in human HuH-7 and HepG2 cells and in the mouse cell line TIB-75. The mouse isoforms of the human GR and PXR and the mouse Shp were included in these assays taking into account the potential species differences of the transcription factor isoforms. *In silico* and *in vitro* assays with the m*Slco1a1* and m*Slco1a4* promoters and treatment of the human and mouse cell lines with ligands for the human and mouse GR/Gr, PXR/Pxr and the human FXR and the mouse Shp analyze a potential interaction of the transcription factors with the promoters.

6 Materials and Methods

6.1 Chemicals, kits and instruments

A list with all chemicals, kits and instruments including their manufacturers can be found at the end of section Materials and Methods.

6.2 Origin and culture of cell lines

Five human colonic (Caco-2, T84, LS 174T, HT-29 and DLD-1), 1 human duodenal (HuTu 80), 2 human hepatic (Hep G2, HuH-7), 1 mouse small intestinal (IEC4.1), 1 mouse hepatic (TIB-75) and 2 rat small intestinal (IEC-6, IEC-18) cell lines served as epithelial *in vitro* models. All human cell lines were routinely subcultivated in the lab. The rat cell lines were bought from LGC Promochem, Molsheim, France. The cell line IEC4.1 was a kind gift from Dr. A. Jevnikar from the Division of Nephrology, University of Ontario in Canada, whereas the cell line TIB-75 was generously provided by Dr. R. Graf from the Division of Visceral and Graft Surgery of the University Hospital of Zurich. All cell lines were handled and subcultivated according to the ATCC product information and in the case of IEC4.1 according to Li and co-workers (Li et al., 1997).

6.3 RNA extraction

Total RNA extraction from cells and rodent tissue samples was achieved by using the guanidine isothiocyanate and phenol containing reagent TRIzol. All samples were collected in 1.5 ml Safe-Lock tubes. TRIzol in quantities of 500 µl or 1000 µl were applied to extract RNA from either 6-well, 12-well and 24-well Corning multiple well plates, 35- and 100-mm Corning dishes and specimens of mouse and rat tissue. Rat and mouse tissue explants from liver, kidney and the intestine were processed in 1.5 ml Safe-Lock tubes and broken by several passages through 18G x 1

½ ‘‘, 21G x 1 ½ ‘‘ and 22G x 1 ¼ ‘‘ needles followed by drawing up in a 1-ml U-100 syringe. The tissue pieces were pipetted several times up and down the syringe to be homogenized as far as possible. Adherent cells were directly lysed in their culture dish wells by pipetting up and down and were then transferred to 1.5-ml Safe-Lock tubes. The volumes indicated in the following protocol are needed to extract RNA from a 1000 µl mixture of tissue or cultivated cells both suspended in TRIzol. The volumes were divided into half whenever 500 µl of the TRIzol reagent were used.

First, the homogenized samples were left at room temperature for 5 minutes for dissociation of the nucleoprotein complexes. The samples were vigorously mixed with 200 µl chloroform, incubated at room temperature for 2-3 minutes and centrifuged (12,000 g, 15 min, 4 °C). As in the following steps a microcentrifuge 5417 R was used. As a result, 3 phases are formed. A lower protein, phospholipid and DNA containing phase, a DNA containing interphase and an upper aqueous phase where the RNA remained. The RNA was transferred to a 1.5 ml RNase-free Microfuge tube, and subsequently 500 µl isopropanol were added. The samples were stored at -20 °C for 10 minutes at least and centrifuged (14,000 rpm, 10 min, 4 °C). After removal of the supernatant, the RNA pellet was washed with 500 µl 75% ethanol and centrifuged (5 minutes with 14,000 rpm at 4 °C). The pellet was air-dried for 5-10 min and resuspended in 15-60 µl nuclease-free water depending on the $A_{260/280}$ ratio (see Quantification of DNA/ RNA).

6.4 Plasmid purification from *Escherichia coli*

Medium scale

Cultures of *E. coli* were grown in 50 ml LB broth/Carb overnight for 12-16 h. Plasmid DNA was extracted with the Qiagen Plasmid Midi kit from 50 ml bacterial lysate. The bacterial culture medium was replaced by 4 ml resuspension buffer P1 after centrifugation (4000 rpm, 15 min, 4 °C, centrifuge 5810 R). The cells were lysed for 5 minutes in 4 ml sodium hydroxide containing buffer P2. After neutralization with 4 ml acetic acid buffer P3, the lysate was applied to a previously equilibrated (4 ml buffer QBT) QIAGEN-tip. The plasmid DNA was collected due to gravity flow

through the resin of the column for 10 min. The resin was washed 2x with 10 ml wash buffer QC, and finally the DNA was eluted with 5 ml elution buffer QF into autoclaved plastic tubes filled with 3.5 ml isopropanol. The obtained DNA was precipitated by centrifuging (10,000 rpm, ≥ 30 min, 4 °C, Avanti J-E centrifuge). The pellet was dissolved in 400 µl Tris-EDTA buffer solution pH 7.4 and transferred to 1.5-ml tubes. The DNA was precipitated with 1 ml 100% ethanol and 40 µl sodium acetate solution pH 5.0 at -20 °C for ≥ 30 min. The pellet was then washed with 70% ethanol and resuspended in 50 µl Tris-EDTA buffer solution.

Small scale

Cultures of *E. coli* were grown in 3 ml LB broth/Carb overnight for 12-16 h. The GeneJET Plasmid Miniprep Kit was used to purify plasmid DNA from 3 ml bacterial lysate. The cells were resuspended in 250 µl resuspension solution, lysed with 250 µl lysis solution and subsequently the lysis was stopped by adding 250 µl neutralization solution. The cell debris and chromosomal DNA were pelleted by centrifugation (13,000 rpm, 5 min, 4 °C, microcentrifuge 5417 R). The plasmid containing supernatant was transferred on a GeneJET™ spin column and centrifuged (1 min, 13,000 rpm, 4 °C, microcentrifuge 5417 R). The spin column membrane was washed 2x with 500 µl wash solution with subsequent centrifugations (13,000 rpm, 1 min , 4 °C, microcentrifuge 5417 R). The plasmid DNA was finally eluted with 30 µl elution buffer and centrifugation (13,000 rpm, 2 min, 4 °C, microcentrifuge 5417 R).

6.5 Glycerol stocks of bacterial cells

Glycerol stocks of bacterial cultures were prepared in LB broth/Carb medium supplemented with glycerol (final glycerol concentration was 20% v/v). The cultures were transferred to CryoTube vials and then stored in a liquid nitrogen storage tank.

6.6 DMSO stocks of mammalian cell lines

The cell line specific complete growth media were supplemented with another 10% (v/v) fetal bovine serum and 10% (v/v) dimethyl sulfoxide (DMSO). The cells were suspended in this medium, put into a –80 °C freezer overnight and then stored in a liquid nitrogen storage tank.

6.7 Quantification of DNA/ RNA

The quantities of nucleic acids in all samples were determined by their ability to absorb ultraviolet light at the wavelength of 260 nm (spectrophotometer ND-1000). The purity of the samples was assessed by determination of the $A_{260/280}$ ratio (partial overlapping of the absorption of nucleic acids at 260 nm and proteins at 280 nm). The ratio ranged between 1.8 and 2.0.

6.8 Polymerase chain reaction

Cloning of mammalian promoter sequences into reporter gene expressing vectors or transcription factor genes into expression vectors was done by using two different PCR kits. A typical reaction mix contained the target DNA, two primers complementary to the target DNA, a thermostable DNA polymerase and nucleotides. In most cases the *in vitro* amplification of specific DNA sequences was successful using illustra puReTaq Ready-To-Go PCR beads. However, DNA tends to form secondary structures fixed by hydrogen bonds between the purines and pyrimidines. These structures prevent the annealing of the primers and the binding and movement of the DNA polymerase along the template DNA towards the 5′ end. Especially regions with a high content of guanine and cytosine lead to strong interactions between nucleotides because the hydrogen bonds interact between them. Therefore, a special kit from Clontech Laboratories (Advantage-GC 2 PCR) was used whenever first PCR reactions with the puReTaq Ready-To-Go PCR beads failed to

amplify the region of interest. The advantages of this kit are the use of DMSO and GC-Melt in the buffer to disrupt base-pairing and a special polymerase mix containing *Taq* polymerase, a proofreading polymerase and TaqStart antibodies. The final GC-Melt concentration of a reaction mix differed from 0 to 1.5 M for each DNA region of interest.
The cycling parameters were set as followed:

Ready-To-Go beads:
(3 step cycles)

- 95 °C for 3 min
- 40 cycles
  - 95 °C for 30 s
  - Primer annealing temperature for 30 s
  - 72 °C 1 min/kb target
- 72 °C 1 min/kb target
- +4 °C

Advantage-GC 2 PCR kit:
(2 step cycles)

- 94 °C for 3 min
- 40 cycles
  - 94 °C 30 s
  - 68 °C 1 min/kb target
- 68 °C for 1 min/kb target
- +4 °C

PCR products were visualised on 1% agarose gels stained with either SYBR - Safe DNA gel stain (0.01%, v/v) or GelRed stain (0.01%, v/v). The PCR product chain length was verified by comparison to either DNA molecular weight marker VII or VIII. The exact DNA sequence was analysed commercially.

6.9 Site-directed mutagenesis

During synthesis of a new DNA strand by polymerases it may occur that the sequence differs from the template DNA. To study the activation of these promoter constructs by transcription factors it is crucial, especially if the mutation(s) are within transcription factors response elements, to mutate the strand back to the wild-type template. On the other hand mutations must be inserted into the response elements to study DNA protein interactions in EMSAs and to examine potentially changed

promoter activity in reporter assays. The QuikChange Site-Directed Mutagenesis Kit was used to make the required point mutations. The procedure includes supercoiled double-stranded DNA vector with the promoter region of interest and two synthetic oligonucleotide primers containing the desired point mutations. The oligonucleotide primers, each complementary to opposite strands of the vector, were extended during temperature cycling by *PfuTurbo* DNA polymerase. Due to insertion of the oligonucleotide primers a mutated plasmid was generated. After the PCR, the product was treated with Dpn I. This endonuclease is specific for methylated and hemimethylated DNA and was used to digest the template DNA. *E. coli* usually methylate DNA and therefore the template DNA is susceptible for digestion by Dpn I. The remaining vector DNA with the desired point mutations was then cloned into XL1-Blue supercompetent cells and the plasmid was analyzed by commercial DNA sequencing.

6.10 Purification of PCR products

PCR products were extracted from agarose gels with the QIAquick Gel Extraction Kit. All centrifugation steps were carried out at 13,000 rpm for 1 min, in a microcentrifuge 5417 R. First, the DNA fragment was excised from the agarose gel with a scalpel and weighed. Three volumes of buffer QG were added to 1 volume of the gel (100 mg ~ 100 µl) and incubated at 50 °C until complete dissolution of the gel piece in a thermomixer. The DNA was bound by applying the sample to a QIAquick column and centrifugation. The addition of 0.5 ml of buffer QG and centrifugation removed all traces of agarose left. The column was washed with 0.75 ml of buffer PE, and the DNA was eluted with 100 µl of buffer EB. The DNA was purified and precipitated in 100% ethanol with 3 M sodium acetate for at least 30 min at -20 °C, followed by centrifugation at full-speed for 10 min, on a microcentrifuge 5417 R. After discarding the supernatant, the DNA was washed with 70 % ethanol, centrifuged for 5 min and finally dissolved in 15 µl nuclease free water.

6.11 Cloning of PCR products into vector systems

PCR products were subcloned into different mammalian vectors depending on their function. All PCR products were first ligated with the pGEM-T vector. The 3′-T overhangs at the insertion site of the vector prevent recirculation and provide compatible nucleotides for PCR products synthesised by Taq polymerases which add 3′-A overhangs to the amplified targets. Synthesised promoter sequences were further sublconed in the pGL3 reporter vector containing a coding region for firefly luciferase. The pGL3Basic vector was applied to examine factors or promoter sequences that potentially regulate mammalian gene expression. All PCR strands coding for transcription factors were subcloned into either pcDNA3.1(+) or pSG5 depending on the restriction enzymes used. In both vectors the expression of the subcloned PCR product is under control of the T7 promoter.

6.12 Assessment of the transcriptional activities of the promoter regions

The Luciferase Assay System was applied to study the promoter activities. The basic principle is that the promoter region of a gene of interest controls the expression of a modified coding region for firefly (*Photinus pyralis*) luciferase. Firefly luciferase catalyzes luciferin oxidation to oxyluciferin generating a flash of light. This bioluminescent reaction is quantitatively detected by a luminometer (GloMax-Multi+ microplate multimode reader or Luminoskan Ascent). Cells were transfected in 48-well plates at a confluency of 70% with 3 µl FuGENE HD/µg DNA. Cells were co-transfected with 400 ng pGL3Basic luciferase reporter vectors, 200 ng transcription factor expression plasmids and 100 ng control reporter vectors coding for *Renilla* luciferases (*Renilla reniformis*, pRL-CMV or phRG-tk). 16 hours after the co-transfections, specific ligands for transcription factors were added if necessary. 36-40 hours after transfection, cells were harvested in 1x Passive Lysis Buffer for dual luciferase assays. The term ‘dual’ refers to the normalization of the activity of the experimental reporter to the activity of the internal control minimizing experimental variability caused by cell viability or transfection efficiency. The activities of *Photinus*

and *Renilla* luciferase were sequentially measured from the same 10 µl sample by adding 55 µl Luciferase Assay Reagent II followed by 55 µl Stop & Glo Reagent. Firefly and *Renilla* luciferases have dissimilar enzyme structures and substrate requirements. These differences allow a selective discrimination between their bioluminescent reactions.

6.13 RNA interference

Small interfering RNAs (siRNAs) are double-stranded RNA oligonucleotides that specifically bind target RNA sequences forming the RNA-induced silencing complex (RISC). This complex activates RNases and cleaves RNA, which leads to a decreased gene expression. The lipid reagent siLentFect was applied to deliver siRNA strands into Caco-2 cells. The reagent is a mixture of a cationic compound and a co-lipid. Approximately 500,000 cells/2 ml growth medium were inoculated in wells of a 12-well plate the day before transfection. A mixture containing 5 µl siLentFect and either 40 nM or 80 nM siRNA, topped up to 100 µl with Opti-Mem was incubated for 20 min at room temperature and then added to the cells after removal of the growth medium. The cells were then incubated in a CO_2/RH -incubator at 37 °C for 30 min. Following the addition of 500 µl growth medium, the plates were incubated during 24 hours in the CO_2/RH -incubator. Then, the transfection was repeated as described before and possible ligands for transcription factors were added. 48 hours after the first transfection, cells were harvested in TRIzol for RNA extraction.
The control group was treated with siGENOME non-targeting siRNA pools.

6.14 *In vitro* translation of transcription factors

The TnT T7 coupled reticulocyte lysate system was used for transcription and translation of eukaryotic genes cloned downstream from T7 RNA polymerase promoters. The advantage of this kit is the incorporation of the transcription in the

translation mix which reduces the expenditure of time and work. Furthermore the protein yield is in most cases significantly higher than using standard *in vitro* rabbit reticulocyte lysate-based translations. Typically 1 µg of plasmid DNA was added to an aliquot of the TnT master mix and incubated in a 50 µl reaction volume for 90 minutes at 30 °C.

TnT master mix

25 µl	TnT lysate
2 µl	TnT reaction buffer
1 µl	TnT T7 RNA polymerase
0.5 µl	Amino acid mixture, minus leucine (1 mM)
0.5 µl	Amino acid mixture, minus methionine (1 mM)
1 µl	Rnasin ribonuclease inhibitor (40 U/ µl)

6.15 Electrophoretic mobility shift assay

The ability of nuclear protein extracts to specifically bind a DNA sequence was examined with electrophoretic mobility shift assays. Oligonucleotides were designed to be 27 bases long and had a 5'-AGCT overhang in the top strand and a 5'-GATC overhang in the bottom strand. These overhangs made it possible to label the annealed oligonucleotides with radioactivity by fill-in reactions in addition to sublcone them into the pRL-TK luciferase vector. The oligonucleotides were synthesized and purified via Reversed Phase HPLC carried out by Microsynth AG. They were dissolved in 10 mM Tris-HCl (pH 8.5) to a concentration of 1 mg/ml confirmed by measurement of the absorption. Of each complementary oligonucleotide 10 µg were combined, 1 µl of 5 M NaCl was added and made up to 50 µl with 10 mM Tris-HCl (pH 8.5). The oligonucleotides were then put in a shaking thermomixer at 95 °C for 5 min, then at 65 °C for 10 min and at last at 37 °C for 10 min. After this incubation, 3 µl of 5 M NaCl were added, and the oligonucleotides were diluted to a final concentration of 1 µg/µl with 10 mM Tris-HCl (pH 8.5) confirmed by absorption measurement. The annealed oligonucleotides (50 ng) were

labeled in reactions containing 1 µl of MultiScribe reverse transcriptase (50 U/µl) or Superscript II (200 U/µl), 10.5 µl ddH_2O, 2 µl 0.1 M DTT, 1 µl 5 mM dGTP/dCTP/dTTP, 1 µl α-^{32}P-dATP solution (20 mCi/ml, 6000 Ci/mmol) and the reverse transcriptase specific buffer to a final volume of 20 µl. These reaction mixtures were incubated at 37 °C for 30 min, 30 µl of ddH_2O were added and applied to MicroSpin G-25 columns to remove unincorporated nucleotides. They were spun down at 750 x g for 2 min, and the labeled probes were collected. Ten µg of nuclear cell extracts prepared using the NE-PER extraction kit, or 1.5 µl of in vitro translated transcription factor proteins generated with the TnT T7 coupled reticulocyte lysate system were used per DNA-binding reaction. Protein-DNA complexes were formed in the binding buffer (20 mM Tris-HCl pH 8.0, 60 mM KCl, 2 mM $MgCl_2$, 12% (v/v) glycerol, 0.3 mM DTT, 87.5 ng/µl preboiled poly(dI-dC)-poly(dI-dC)) in a total volume of 20 µl. 50,000 cpm of the labeled probe were added to the reaction and incubated for 10 min at 30 °C. In supershift experiments, 1 µl of the corresponding antibody was added to the reaction 1 h before the labeled probe and incubated at 4 °C. The samples were loaded onto pre-electrophoresed 5% native acrylamide gels (per gel: 6.25 ml 40% acrylamide, 4.1 ml 2% bisacrylamide, 2.5 ml 10x TBE, 500 µl 10% APS, 50 µl TEMED, 36.6 ml ddH_2O) and run at 200 V in 0.5x diluted TBE buffer for approximately 3 hours. The gels were then fixed in 10% (v/v) acetic acid for 10 min, rinsed with water, dried onto Whatman DE81 paper under vacuum, and exposed to Kodak BioMax MR-1 films at -80°C.

6.16 Extraction of nuclear and cytoplasmic proteins

The extraction of nuclear and cytoplasmic proteins from cultured cells was achieved by use of the NE-PER Nuclear and Cytoplasmic extraction kit. The stepwise process involves lysis of the cells, separation of the cytoplasm from the intact nuclei and extraction of the proteins from the DNA and RNA of the nuclei. The following steps refer to extract the proteins from cells cultivated in a 10-cm Corning dish. Cells were washed with 5 ml PBS and scraped into 1 ml ice-cold PBS containing 1x Complete solution. After a spin at 700 x rcf at 4 °C for 2 min (microcentrifuge 5417 R)

and removal of the supernatant, 150 µl cytoplasmic extraction reagent I (CER I containing 1x Complete solution) was added and the tube was vortexed for 5 s. The addition of the cytoplasmic extraction reagent II (CER II) was followed by 5 s vortexing, 2 min incubation on ice and again 5 s vortexing. The sample was centrifuged for 4 min at 14,000 x rcf at 4 °C, (microcentrifuge 5417 R). The supernatant containing the cytoplasmic proteins was transferred to a new tube and stored. The remaining pellet was resuspended in 25 µl nuclear extraction reagent (NER) containing 1x Complete solution, vortexed for 5 s, incubated on ice for 20 min and vortexed again for 5 s. Following a centrifugation at maximum speed for 10 min at 4 °C and transfer of the supernatant nuclear extract to a new tube, a second nuclear extraction with NER was performed as described above and both nuclear extracts were combined. The protein concentration of all samples was determined with an assay based on the use of bicinchoninic acid.

6.17 BCA protein assay

Protein concentrations were determined by spectrophotometric detection based on bicinchoninic acid (BCA) using the BCA protein assay kit. The method combines the reduction of Cu^{2+} to Cu^{1+} by proteins in an alkaline medium (Biuret reaction) with the spectrophotometric detection of Cu^{1+} using a reagent containing BCA. The complex of two BCAs and one Cu^{1+} ion shows a strong absorbance at 562 nm. All protein concentrations were determined with reference to bovine γ-globuline. A series of bovine γ-globuline concentrations ranging from 0.025 mg/ml to 1 mg/ml were assayed before the concentration of each unknown was determined based on the standard curve. Nuclear extract concentrations were determined in 2 µl or 4 µl sample volumes, whereas the protein determination in transport experiments was performed with 12.5 µl of lysates from 35-mm dishes. All samples were equalized with regard to the buffers used in nuclear extracts or transport experiments to a total volume of 50 µl. Two hundred µl of the working reagent containing the BCA reagent A and reagent B (50:1, v/v) was added and incubated at

37 °C for 30 min. The absorption at 550 nm was measured with a spectrophotometer (GloMax-Multi+ microplate multimode reader).

6.18 Western blot

To detect specific proteins in a RIPA buffer extract of cultivated cells, gel electrophoresis was applied. After removing the secondary or tertiary structures with reducing agents, the proteins of a sample were separated by their molecular weight. The separating gel was prepared by mixing 4.4 ml ddH_2O, 2.5 ml resolving gel buffer, 3 ml 30% acrylamide/bis solution, 100 µl 10% APS and 5 µl TEMED. The stacking gel consisted of 3.05 ml ddH_2O, 1.25 ml stacking buffer, 650 µl 30% acrylamide/bis solution, 50 µl 10% APS and 7 µl TEMED. 10 well-combs and 0.75 mm spacers were used in all experiments. Fifteen µg of cell extract were adjusted to a volume of 10 µl with PBS 1x. After addition of 2.5 µl loading buffer, the mix was heated for 10 minutes at 85 °C, spun down and applied into the wells. As protein standard 7 µl of SeeBlue Plus2 were loaded onto the gel. The gel was run first at 300 V for 5 min, then 150 V until the probes reached the separating gel and finally at 200 V. Afterwards the protein was transferred onto a nitrocellulose membrane as following: First, fresh transfer buffer was prepared (3 g TRIZma-base, 14.4 g glycine, 200 ml methanol and ddH_2O to give a final volume of 1 l). The gel was placed on top of the membrane and sandwiched between two stacks of sponges and filter papers. After placing in a tank an ice-cold block was put into the tank to keep the transfer buffer cool. A current of ~250 mA during 1 hour was used to pull the proteins from the gel into the nitrocellulose membrane. The blot was washed with blocking solution (PBS 1x, 5% non fat dry milk, 0.1% Tween 20) by incubation of 1 hour shaking at RT. The incubation with the first antibody varied in time and concentration (more precisely described in the result section). The blot was washed 3 times for 15 minutes with plenty of block solution. The secondary HRP-conjugated antibody incubation varied as well in time and concentration (more precisely described in the result section). The blot was briefly dried and covered with

SuperSignal WestFemto for 5 minutes. The blot was again briefly dried, covered with plastic wrap and exposed to Amersham Hyperfilm ECL (the exposure time varied).

6.19 Chromatin immunoprecipitation (ChIP)

Chromatin immunoprecipitation is a technique which allows to determine whether specific proteins interact with genomic regions. The basis of this technique is to fix chromatin proteins to DNA by treating cells with formaldehyde. The chromatin is then extracted, sheared by sonication into fragments and the proteins associated with DNA are immunoprecipitated with antibodies. The following instructions always refer to a ChIP starting with a 10-cm dish using the ChIP-IT express chromatin immunoprecipitation kit.

6.19.1 Cell fixation

The following solutions were freshly prepared:

1. Fixation solution: 0.45 ml 16% (v/v) formaldehyde to 7 ml minimal cell culture medium, placed at RT
2. 1x PBS: add 0.84 ml 10x PBS to 7.6 ml ddH_2O, placed on ice
3. Glycine Stop Fix solution: combine 361 µl 10x Glycine buffer, 361 µl 10x PBS and 2.9 ml ddH_2O, placed at RT
4. Cell Scraping Solution: add 72 µl 10x PBS to 650 µl ddH_2O, placed on ice. Just before use 3.6 µl 100 mM PMSF were added

After removal of the growth medium, the cells were fixed with the fixation solution on a shaking platform during 10 min. The fixation solution was sucked off and the cells were washed with 1x PBS solution. The fixation reaction was then stopped by adding the Glycine Stop Fix solution rocking for 5 min. The cells were again washed with 1x PBS solution after pouring off the Glycine Stop Fix solution. The cells were scraped into the Cell Scraping Solution and transferred to a tube. The cells were centrifuged (2,500 rpm, 10 min, 4 °C, microcentrifuge 5417 R) and the supernatant was removed.

6.19.2 Shearing by sonication

The pellet was resuspended in 1 ml ice-cold Lysis Buffer supplemented with 5 µl PIC and PMSF and incubated for 30 min on ice. The cells were transferred to an ice-cold dounce homogenizer and the nuclei were released with 10 strokes. The suspensions were pipetted into 1.7-ml siliconized tubes and centrifuged (5,000 rpm, 10 min, 4 °C) to pellet the nuclei. The supernatant was discarded and the nuclei resuspended in 350 µl Shearing Buffer supplemented with 1.75 µl PIC and placed on ice. The DNA was sheared with the Branson Digital sonifier at power setting 25% with a pause of 30 s between each pulse: The nuclei from Caco-2 were sonified with 5 pulses, whereas Huh7 needed 10 pulses. The sheared chromatin was centrifuged (15,000 rpm, 10 min, 4 °C, microcentrifuge 5417 R). The supernatant was transferred to a new 1.7-ml siliconized tube. 15 µl from each sample was aliquoted as the 'input DNA'.

6.19.3 Immunoprecipitation

For the precipitation step, 50 µl aliquots of sheared chromatin were incubated without any antibody, with 1 µg of negative control anti-serum mouse IgG1 or with 1 µg of the antibodies raised against the transcription factors of interest. Twenty five µl of protein G magnetic beads, 10 µl ChIP buffer and 1 µl PIC were added to the reaction mixture and ddH_2O was added to a final volume of 100 µl. The reaction mixtures were incubated on an end-to-end rotator at 4 °C overnight.

6.19.4 Wash of the magnetic beads

The tubes were placed on a magnetic stand and the supernatants carefully removed. The beads were then washed 1x with 800 µl ChIP buffer I and 2x with 800 µl ChIP buffer II. After the washing, as much supernatant as possible was removed.

6.19.5 Elution of chromatin, reversion of cross-links and treatment with proteinase K

The beads were resuspended with 50 µl elution buffer AM2 and incubated for 15 min in a thermomixer at RT. To elute the chromatin, 50 µl of the reverse cross-linking buffer was added. The tubes were placed in a magnetic stand and the supernatant containing the chromatin was transferred to fresh tubes. To 10 µl of the 'input DNA' sample, 88 µl ChIP buffer 2 and 2 µl 5 M NaCl were added. The ChIP and the 'input DNA' samples were heated at 95 °C for 15 min, cooled down to RT and supplemented with 2 µl proteinase K. The samples were digested at 37 °C during 1 h followed by addition of 2 µl proteinase K stop solution. Two amplicons were assayed by immunoprecipitation tests, using puReTaq Ready-To-Go pcr beads and the oligonucleotides of interest. Oligonucleotides were designed to amplify the transcription factor DNA response element. As negative control served oligonucleotides amplifying a region from an intron of the gene of interest.
After the initial denaturation stage at 94 °C for 3 min, the PCR cycling conditions were 94 °C for 20 s, 60 °C for 30 s, and 72 °C for 1 min. Samples were taken from the 30^{th}, 35^{th} or 40^{th} cycle. 5 µl of each PCR product were resolved on 1.5% agarose gels and detected with SYBR - Safe DNA gel or GelRed stain.

6.20 Reverse transcription of RNA

The High Capacity cDNA Reverse Transcription kit was used for reverse transcription of total RNA to single-stranded cDNA. Between 1 and 2 µg of RNA were reverse transcribed in 20 µl reaction volumes. Two µl of 10x RT buffer, 0.8 µl of 25x dNTP mix, 2 µl 10x RT random primers, 1 µl MultiScribe reverse transcriptase, 1 µl RNase inhibitor and 3.2 µl of ddH_2O were combined with the respective amount of RNA in 10 µl ddH_2O and incubated in a thermomixer as follows: 10 min at 25 °C, 120 min at 37 °C and 5 min at 85 °C.

6.21 *Ex vivo* treatment of rodent tissue specimens with ligands of transcription factors

Tissue samples of rodents were collected just after euthanasia and placed immediately in ice-cold PBS. Small tissue pieces were placed into pre-warmed DMEM supplemented with agonists or antagonists of transcription factors of interest. The samples were incubated during 4 hours in a CO_2/RH incubator at 37 °C. The RNA of the samples was isolated following the protocol 'RNA extraction'.

6.22 Statistical analysis

All cell-based experiments were repeated at least three times (except the co-transfection assays of the m*Slco1a1* and m*Slco1a4* promoter constructs, which were done only once). In the present thesis representative experiments are shown. All data from luciferase assays and real-time PCR measurements were analysed as means ± SDs. The values of each experimental group were analysed by one-way analysis of variance (ANOVA) followed by the Tukey post hoc test. P values < 0.05 were considered as significant. The statistical values were calculated based on the software GraphPad Prism.

6.23 List of chemicals / kits / equipment

α-^{32}P-dATP (20 mCi/ml, 6000 Ci/mmol) (PerkinElmer, Waltham MA, USA)

40% acrylamide (Bio-Rad, Hercules CA, USA)

30% acrylamide/bis solution (Bio-Rad, Hercules CA, USA)

2% bisacrylamide (Bio-Rad, Hercules CA, USA)

9-cis-RA (10 mM in DMSO) (Sigma-Aldrich, Saint-Louis, Missouri MO, USA)

6-/ 12-/ 24-/ 48-well Corning multiple well plates (Corning, Corning NY, USA)

100 mm Corning dishes (Corning, Corning NY, USA)

15-deoxy-Δ12,14-prostaglandin J2 (10 mM in DMSO) (Sigma-Aldrich, Saint-Louis, Missouri MO, USA)

7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, California CA, USA)

18G x 1 ½ ‘‘, 21G x 1 ½ ‘‘, and 22G x 1 ¼ ‘‘ needles (Terumo, Tokyo, Japan)

1.5 ml RNase-free Microfuge tubes (Ambion/ Applied Biosystems, Foster City, California CA, USA)

1.5 ml Safe-Lock tubes (Vaudaux-Eppendorf , Hamburg, Germany)

1 ml U-100 syringes (Nipro, Osaka, Japan)

Advantage-GC 2 PCR (Clontech Laboratories/ Takara Holding Company Inc, Kyoto, Japan)

Agarose for routine use (Sigma-Aldrich, Saint-Louis, Missouri MO, USA)

AHPN (10 mM in DMSO) (Sigma-Aldrich, Saint-Louis, Missouri MO, USA)

Amersham Hyperfilm ECL (GE Healthcare, Fairfield CT, USA)

Anti-serum mouse IgG1 (Dako Denmark A/S, Glostrup, Denmark)

APS ammonium persulfate (10% w/v) (Sigma-Aldrich, Saint-Louis, Missouri MO, USA)

Avanti J-E Centrifuge (Beckman Coulter, Brea, California CA, USA)

BCA protein assay kit (Thermo Fisher Scientific, Waltham MA, USA)

Bovine γ-globuline (Bio-Rad, Hercules CA, USA)

Branson Digital sonifier (Branson, Ultrasonics, Danbury CT, USA)

Carbenicillin (stock 50 mg/ml; final concentration 50 µg/ml) (Novagen/ Merck, Whitehouse Station, New Jersey NJ, USA)

CDCA (100 mM in DMSO) (Sigma-Aldrich, Saint-Louis, Missouri MO, USA)

Centrifuge 5810 R (Vaudaux-Eppendorf AG, Hamburg, Germany)

ChIP-IT express chromatin immunoprecipitation kit (Active Motif, Carlsbad, California CA, USA)

Ciprofibrate (30 mM in DMSO) (Sigma-Aldrich, Saint-Louis, Missouri MO, USA)

Complete, Mini protease inhibitor cocktail tablets (Hoffmann-La Roche, Basel, Switzerland)

CryoTube vials (Nunc, Roskilde, Denmark)

Dexamethasone (1 mM in EtOH) (Sigma-Aldrich, Saint-Louis, Missouri MO, USA)

DNA molecular weight marker VII and VIII (Hoffmann-La Roche, Basel, Switzerland)

Escherichia coli (Life Technologies, Carlsbad CA, USA and unknown origin)

Ethanol for molecular biology, assay: ≥ 99.8% (Sigma-Aldrich, Saint-Louis, Missouri MO, USA)

Fetal bovine serum gold (PAA Laboratories, Pasching. Austria)

Formaldehyde (Thermo Fisher Scientific, Waltham MA, USA)

FuGENE HD (Hoffmann-La Roche, Basel, Switzerland)

GelRed stain (Chemie Brunschwig, Basel, Switzerland)

GeneJET Plasmid Miniprep Kit (Fermentas/ Thermo Fisher Scientific, Waltham MA, USA)

GloMax-Multi+ microplate multimode reader (Promega, Madison WI, USA)

Glycine (Sigma-Aldrich, Saint-Louis, Missouri MO, USA)

GraphPad Prism software (GraphPad, San Diego CA, USA)

GW-9662 (100 mM in DMSO) (Enzo Biochem, Farmingdale NY, USA)

GW-1929 (40 mM in DMSO) (Sigma-Aldrich, Saint-Louis, Missouri MO, USA)

High Capacity cDNA Reverse Transcription (Invitrogen, Carlsbad, California CA, USA)

Hyperforin (0.25mg/ml) (Sigma-Aldrich, Saint-Louis, Missouri MO, USA)

illustra MicroSpin G-25 columns (GE Healthcare, Fairfield CT, USA)

illustra puReTaq Ready-To-Go pcr beads (GE Healthcare, Little Chalfont, United Kingdom)

Isopropanol for molecular biology, assay ≥ 99% (Sigma-Aldrich, Saint-Louis, Missouri MO, USA)

Formaldehyde 16% (Thermo Fisher Scientific, Waltham MA, USA)

Kodak BioMax MR-1 (PerkinElmer, Waltham MA, USA)

LB agar (Sigma-Aldrich, Saint-Louis, Missouri MO, USA)

LB broth EZMix (Sigma-Aldrich, Saint-Louis, Missouri MO, USA

Loading buffer: 95 µl 5 x see blue and 5 µl β-mercaptoethanol

Luciferase Assay System (Promega, Madison WI, USA)

Luminoskan Ascent (Thermo Fisher Scientific, Waltham MA, USA)

Methanol (Sigma-Aldrich, Saint-Louis, Missouri MO, USA)

Microcentrifuge 5417 R (Vaudaux-Eppendorf AG, Hamburg, Germany)

Mifepristone (50 mM in EtOH) (Sigma-Aldrich, Saint-Louis, Missouri MO, USA)

MK-886 (40mM in DMSO) (Enzo Biochem, Farmingdale NY, USA)

NE-PER nuclear and cytoplasmatic extraction kit (Thermo Fisher Scientific, Waltham MA, USA)

Nitrocellulose membrane (Bio-Rad, Hercules CA, USA)

Non-fat dry milk (Bio-Rad, Hercules CA, USA)

Nuclease-free water (Thermo Fisher Scientific, Waltham MA, USA)

Opti-Mem reduced serum media (Invitrogen, Carlsbad, California CA, USA)

PBS (Invitrogen, Carlsbad, California CA, USA)

PCN (2.6 mM in EtOH) (unknown origin)

Penicillin/ streptomycin in 0.85% saline (Gibco/ Invitrogen, Carlsbad, California CA, USA)

poly(dI-dC)-poly(dI-dC) acid sodium salt (Sigma-Aldrich, Saint-Louis, Missouri MO, USA)

Qiagen Plasmid Midi Kit (Qiagen, Hilden, Germany)

QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany)

QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, California CA, USA)

Resolving gel buffer (Bio-Rad, Hercules CA, USA)

See-Blue Plus2 pre-stained protein standard (Invitrogen, Carlsbad, California CA, USA)

siGENOME non-targeting siRNA pool (Thermo Fisher Scientific, Waltham MA, USA)

siLentFect (Bio-Rad, Hercules CA, USA)

Sodium acetate solution pH 5.0 (Sigma-Aldrich, Saint-Louis, Missouri MO, USA)

Spectrophotometer ND-1000 (NanoDrop, Wilmington DE, USA)

Stacking gel buffer (Bio-Rad, Hercules CA, USA)

SuperSignal WestFemto (Thermo Fisher Scientific, Waltham MA, USA)

SYBR - Safe DNA gel stain (Invitrogen, Carlsbad, California CA, USA)

TBE buffer 10x (Thermo Fisher Scientific, Waltham MA, USA)

TEMED N,N,N',N'-Tetramethylethylendiamin (Merck, Darmstadt, Germany)

Thermomixer comfort (Vaudaux-Eppendorf , Hamburg, Germany)

TipOne filter tips (10 µl, 20 µl, 200 µl, 1000 µl) (Starlab, Brussels, Belgium)

TnT T7 coupled reticulocyte lysate system (Promega, Madison WI, USA)

TRIZma (Sigma-Aldrich, Saint-Louis, Missouri MO, USA)

Tris-EDTA buffer solution pH 7.4 (Fluka/ Sigma-Aldrich, Saint-Louis, Missouri MO, USA)

TRIzol (Invitrogen, Carlsbad, California CA, USA)

Troglitazone (30 mM in DMSO) (Enzo Biochem, Farmingdale NY, USA)

Tween 20 (Merck, Whitehouse Station, New Jersey NJ, USA)

Whatman DE81 paper (GE Healthcare, Fairfield CT, USA)

WY-14643 (100 mM in DMSO) (Merck KgaA, Darmstadt, Germany)

7 Results

7.1 The peptide transporter 1 (PEPT1)

All methodologies applied in this study are listed and described in the section Materials and Methods.

7.1.1 *In silico* identification of putative PPAR response elements in human and rodent *PEPT1/Pept1* promoters

The peptide transporter PEPT1 mediates the uptake of di-/tri-peptides and peptidomimetic drugs in the small intestine. PEPT1 is also aberrantly expressed in the colon of patients suffering from inflammatory bowel disease (IBD) (Merlin et al., 2001). In addition the peroxisome proliferator-activated receptor γ (PPARγ), a ligand-dependent transcription factor, plays an important role in IBD etiology. As the ability of PPARγ to regulate human/rodent *PEPT1/Pept1* genes remained unelucidated, *PEPT1/Pept1* promoter activities were studied in intestine-derived human and rodent cell lines.

In silico analysis of human and rodent *PEPT1/Pept1* promoters revealed several putative peroxisome proliferator-activated receptor response elements (PPARs) within around 5000 base pairs towards the 5'-end of the promoter (NCBI accession numbers: NW_925517, human promoter; NW_001030563, mouse promoter; NW_001084709, rat promoter). Two web-based programs, MatInspector (www.genomatix.de) and NUBIScan (www.nubiscan.unibas.ch), identified several putative PPAR response elements (PPREs). Especially focused on PPREs close to the transcriptional start site, two human, four mouse and one rat PPRE were identified. To examine the functionality of the PPREs, promoter constructs of different lengths were cloned into the reporter assay vector pGL3Basic. Three human promoter constructs containing PPREs (*PEPT1* -2010/+57, *PEPT1* -1288/+57, *PEPT1* -506/+57) and one without any PPRE (*PEPT1* -65/+57) were used for co-transfections and

EMSAs. One construct covering the PPREs (m*Pept1* -2150/+6, r*Pept1* -1943/+20) and one without any PPREs (m*Pept1* -1095/+6, r*Pept1* -1048/+20) were cloned from the rodent *Pept1* promoters (Fig. 1).

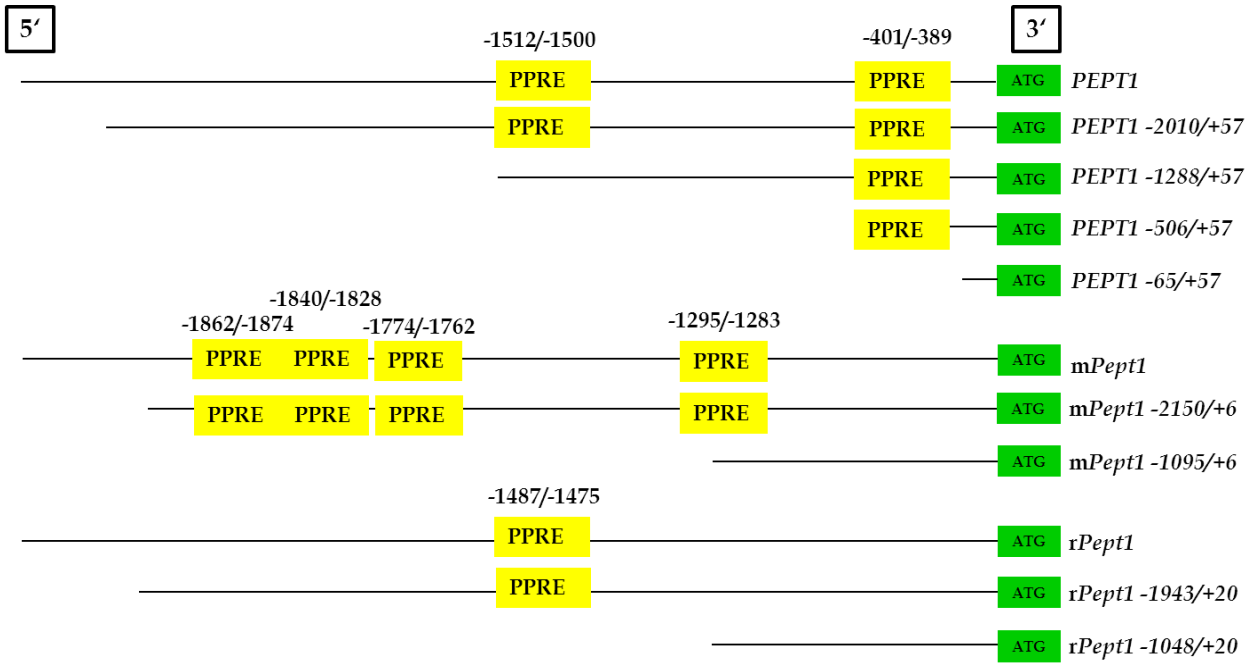


Fig. 1: Human and rodent *PEPT1/Pept1* promoter sequences and constructs. The parts of the promoters cloned into luciferase reporter vectors are indicated by their terminal nucleotides. The 5′-end and the 3′-end of the cloned constructs are indicated with -/+ in relation to the start of transcription. The longest shown human or rodent promoter sequences represent the respective templates used to clone the constructs. Yellow boxes: PPREs, green boxes: start of translation (ATG).

The PPARs bind as heterodimers with the retinoid X receptor alpha (RXRα) to the DNA (PPAR:RXRα). In mammalians, the preferred consensus DNA response element is a direct repeat of two hexanucleotides spaced by one nucleotide, AGGTCAnAGGTCA (Kliewer et al., 1992) (Fig. 2). The potential PPRE in the human and rodent *PEPT1/Pept1* promoters are listed in Figure 2.

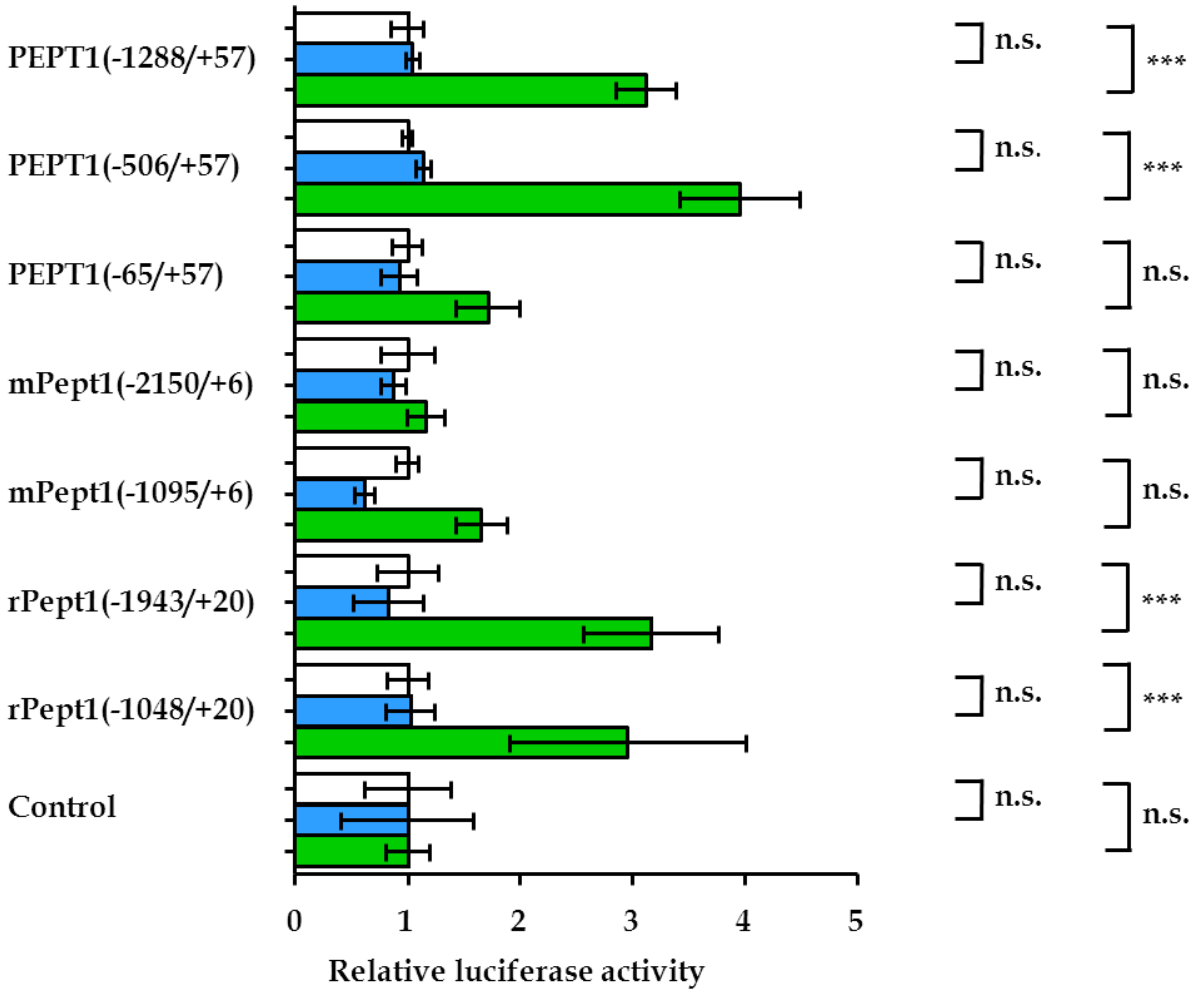
PPAR:RXRα consensus:	A G G T C A n A G G T C A
PEPT1 wt -389/-401:	G **G G** A **C A** g T **G G T C A**
PEPT1 wt -1500/-1512:	T **G G** G **C A** a **A G G** A T **A**
m*Pept1* wt -1283/-1295:	T T A C **C** C t T **G** A A **C** T
m*Pept1* wt -1762/-1774:	T **G** T **T C** T g T **G** T C **C** T
m*Pept1* wt -1828/-1840:	T **G** T C **C** T c T A C A **C** T
m*Pept1* wt -1862/-1874:	T C T **T C** T t T **G** A C **C** T
r*Pept1* wt -1475/-1487:	T **G** A A **C** T t T **G** T G **C** T

Fig. 2: Identification of putative PPREs within the human and rodent *PEPT1/Pept1* promoters. Identical nucleotides compared to the PPAR:RXRα consensus sequence are shown in bold letters. The location of the PPREs is indicated relative to the transcriptional start site.

7.1.2 Transactivation of the human and rat *PEPT1/Pept1* promoters by PPARα and PPARγ

Different human and rodent promoter constructs were used to study whether the contained PPREs mediate the PPAR:RXRα-dependent activation in Caco-2 cells. Final concentrations of 30 µM ciprofibrate, 10 µM 15-deoxy-Δ12,14-prostaglandin J_2 (15d-PGJ2), and 1 µM 9-cis-retinoic acid (9-cis-RA) were used as agonists for PPARα,

PPARγ and RXRα receptor proteins, respectively. Two human *PEPT1* promoter constructs covering one PPRE and a human *PEPT1* promoter part without a putative PPRE were transiently transfected. The two mouse promoter constructs covered four or no PPRE. The rat promoter was examined with two promoter constructs containing a PPRE and one construct lacking the putative PPRE.
Both *PEPT1* (-506/+57) and *PEPT1* (-1288/+57) promoter fragments were potently activated by PPARγ: RXRα, but not PPARα: RXRα. The short *PEPT1* (-65/+57) construct did not mediate a significant luciferase activity by both PPARα:RXRα and PPARγ:RXRα. The m*Pept1(-2150/+6)* construct was not transactivated by either heterodimer in contrast to the weak activation of m*Pept1(-1095/+6)* by PPARγ:RXRα. The two rat promoter fragments were both significantly ($p < 0.001$) activated by PPARγ:RXRα but not by PPARα:RXRα (Fig. 3).



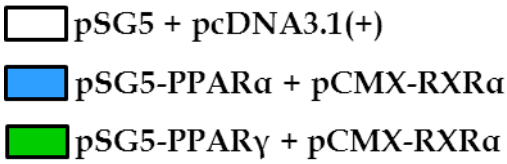


Fig. 3: Co-transfections of Caco-2 cells with *PEPT1/Pept1* promoter constructs and PPARα, PPARγ and RXRα expressing plasmids.. Caco-2 cells were co-transfected with reporter-linked human and rodent *PEPT1/Pept1* promoter constructs (nucleotide numbering relative to the transcription start site), expression plasmids coding for RXRα, PPARα and PPARγ and their respective ligands 9-cis-RA, ciprofibrate and 15d-PGJ2. The relative luciferase activities are shown relative to the cells transfected with pSG5 and pcDNA3.1(+) (white bars). Blue bars represent the relative luciferase activities of cells co-transfected with pSG5- PPARα and pCMX-RXRα, whereas green bars represent co-transfections with pSG5- PPARγ and pCMX-RXRα. ***, $p < 0.001$, *, $p < 0.1$, n.s., not significant.

7.1.3 Binding of *in vitro* translated PPARγ proteins to human and rat PPREs

To study the potential interaction of PPARγ and RXRα with the human and rodent PPREs in vitro, electromobility shift assays (EMSAs) were performed. Oligonucleotides comprising the wild-type human and rodent PPAR binding sites were synthesised and radio-labelled. The mammalian expression vector pSG5, pSG5-PPARγ and pCMX-RXRα were *in vitro* translated with the TnT T7 coupled reticulocyte lysate system. Radiolabeled wild-type PPREs were incubated with the proteins and the complexes formed are shown in figure 4. The interaction of PPRE *PEPT1* wt -1500/-1512 and PPARγ:RXRα was stronger than the binding of *PEPT1* wt -389/-401 to the heterodimer. None of the mouse PPREs was able to bind the protein heterodimers. The rat PPRE showed the strongest interaction between the DNA and PPARγ:RXRα. As a positive control, the interaction of the known PPRE of the human

apical sodium-dependent bile acid transporter ASBT and PPARγ:RXRα was used (Jung et al., 2002).

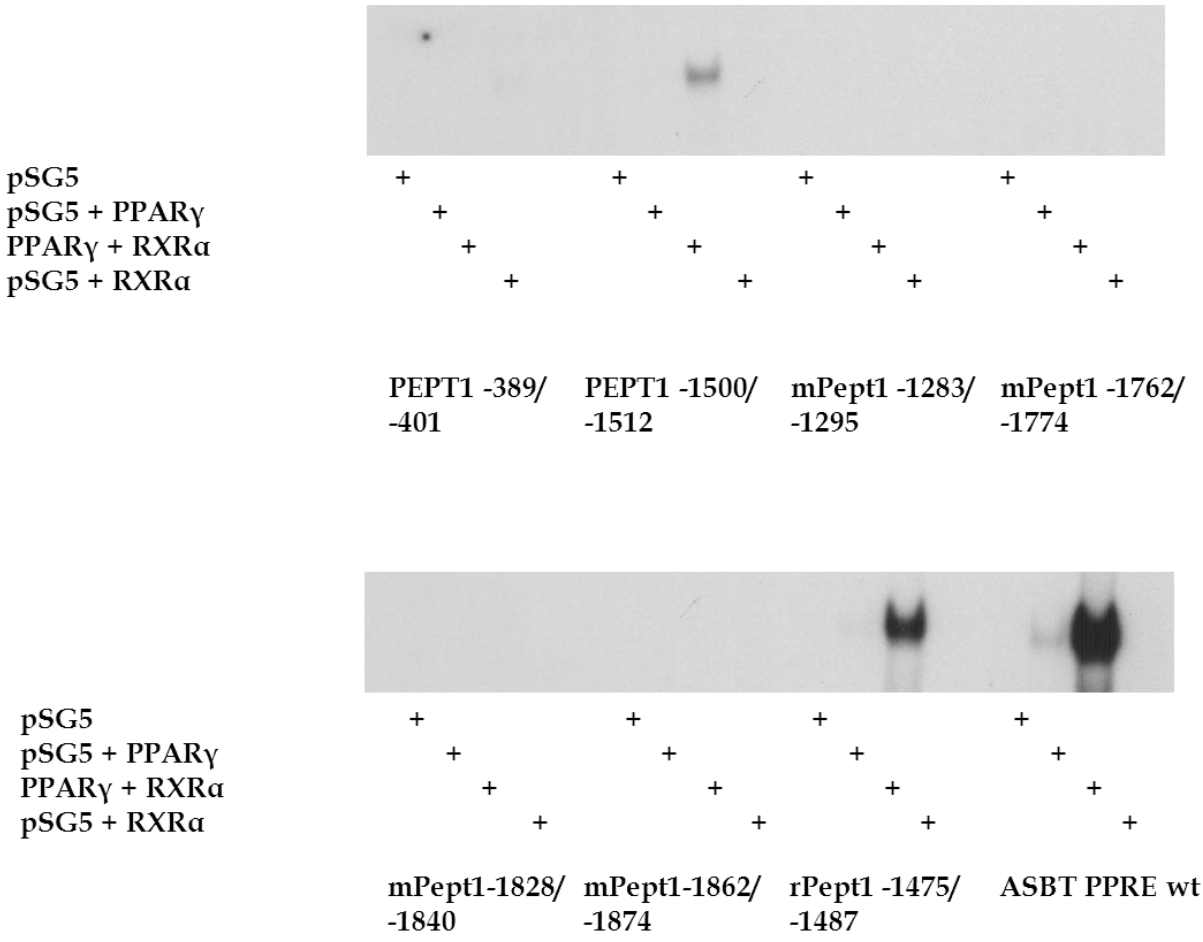


Fig. 4: Electromobility shift assays to show the specific binding of *in vitro* translated PPARγ proteins to human and rat PPREs. EMSAs were performed with *in vitro* translated PPARγ proteins and the *PEPT1*/*Pept1* promoter sequences containing the wild-type PPARγ consensus binding motif as radiolabelled probes. The numbering of the human and rodent PPREs is relative to the transcription start site.

7.1.4. *PEPT1/Pept1* promoter constructs featuring wild-type and mutated PPRE transactivation by PPARγ:RXRα

To reduce the binding of PPARγ:RXRα, site-directed mutagenesis of all predicted human and rat PPREs was applied. The preferred DNA sequence for

PPARγ:RXRα binding was examined by Okuno and co-workers (2001) with PCR-mediated random site selection. The nucleotides to be mutated were chosen according to their studies. The wild-type PPREs were mutated as following with the site-directed mutagenesis kit and further cloned into the reporter vector pGL3Basic (mutated oligonucleotides are red labelled):

PEPT1 wt -389/-401: GGGACAgTGGTCA
mut: GTGACGGTTGGCA

PEPT1 wt -1500/-1512: TGGGCAaAGGATA
mut: TAGGCCAAAAATA

r*Pept1* wt -1475/-1487: TGAACTtTGTGCT
mut: TGCAATTCGTGAT

Three human *PEPT1* promoter constructs with either one or two mutated PPREs and the rat *Pept1* promoter with the mutated PPRE were transiently transfected into Caco-2 cells. The activity of the mutated human promoters was compared to a longer construct of the human *PEPT1* promoter than in figure 3. The same final concentrations of 10 µM 15d-PGJ2, and 1 µM 9-cis-RA were used as agonists for the nuclear receptors PPARγ and RXRα. The human wild-type *PEPT1* promoter and the three human *PEPT1* constructs with mutated PPREs were activated significantly by PPARγ:RXRα. In contrast to the mutated form the wildtype rat *Pept1* promoter construct was not transactivated (Fig. 5).

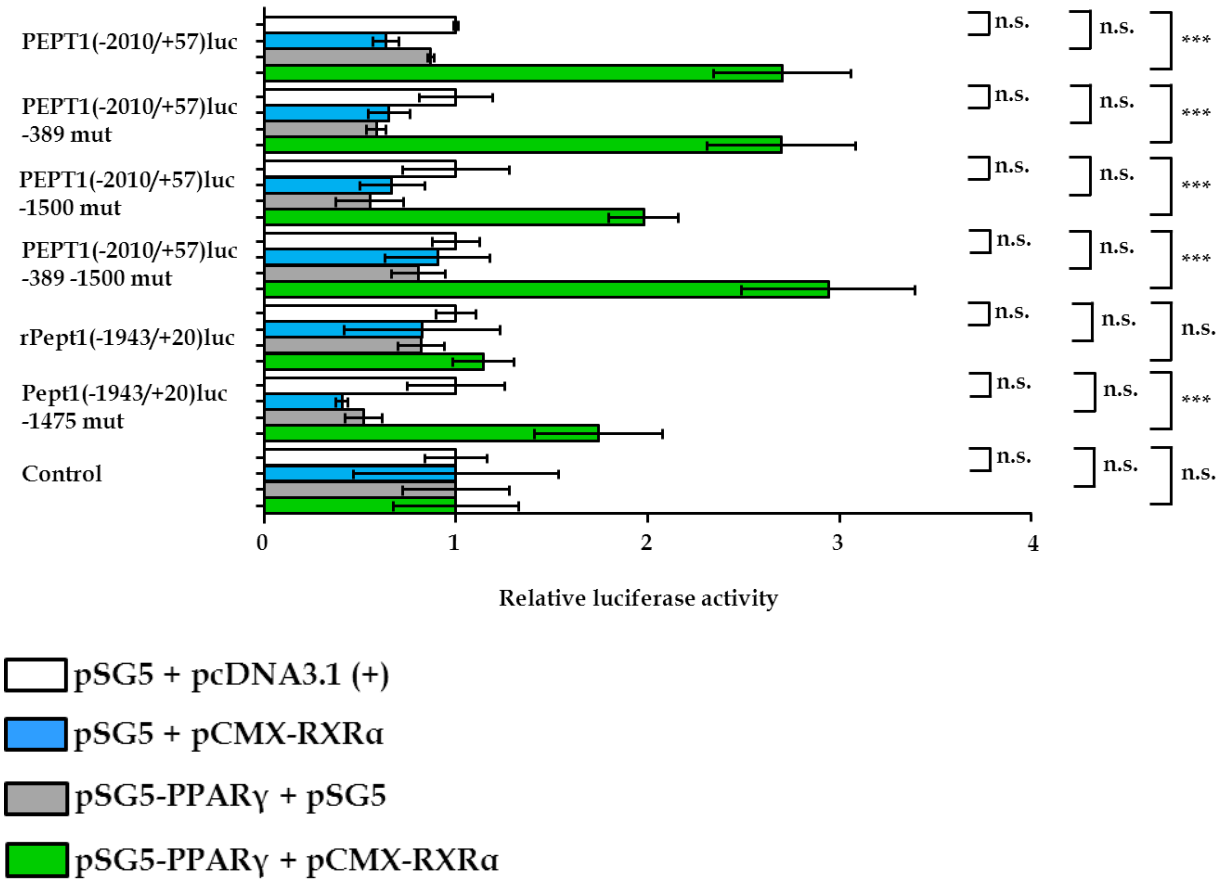


Fig. 5: Caco-2 cells co-transfected with reporter-linked human and rodent *PEPT1/Pept1* promoter constructs (nucleotide numbering relative to the transcription start site) with either wild-type or mutated PPREs. The expression plasmids featuring RXRα and PPARγ constructs and their respective ligands 9-cis-RA and prostaglandin J2 were added as described above. The relative luciferase activities are shown relative to the cells transfected with pSG5 and pcDNA3.1(+) (white bars). Blue bars represent the relative luciferase activities of cells co-transfected with pSG5 and pCMX-RXRα, grey bars represent co-transfections with pSG5-PPARγ and pCMX-RXRα and green bars show the relative luciferase activities of cells co-transfected with pSG5-PPARγ and pSG5. ***, $p < 0.001$, n.s., not significant.

7.1.5 Relative PEPT1 mRNA quantification with real-time PCR in human cell lines

To study the relevance of PPARγ:RXRα in the regulation of the intestinal PEPT1 expression, *in vivo* relative mRNA levels of 5 human colon derived cell lines were measured with real-time PCR. The expression level in HT-29 cells was set to 1 and all other cell lines were compared relative to this. The cycle threshold (Ct) of PEPT1 was always normalized to the Ct of the housekeeping gene beta-actin in all cell lines. In Caco-2 cells, the Ct for PEPT1 was 25 and in T84 cells 32. In all other cell lines the Ct values were above 35 (Fig. 6).

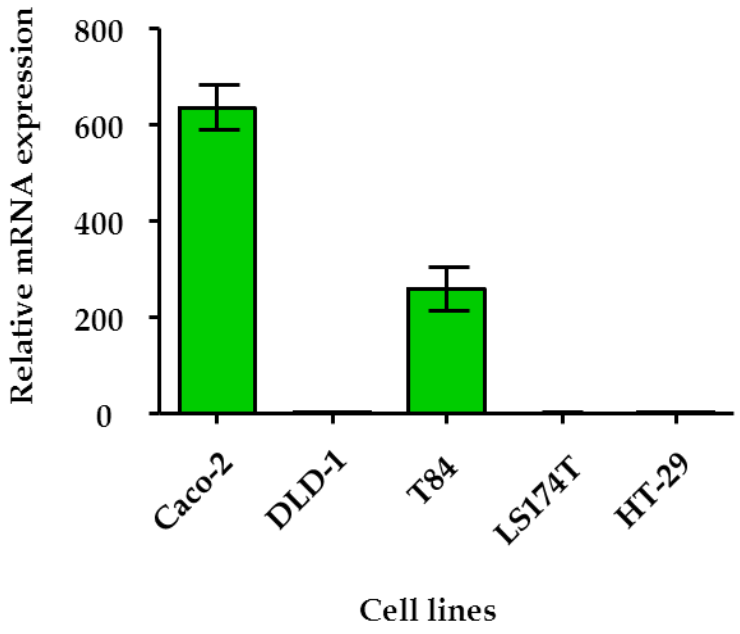
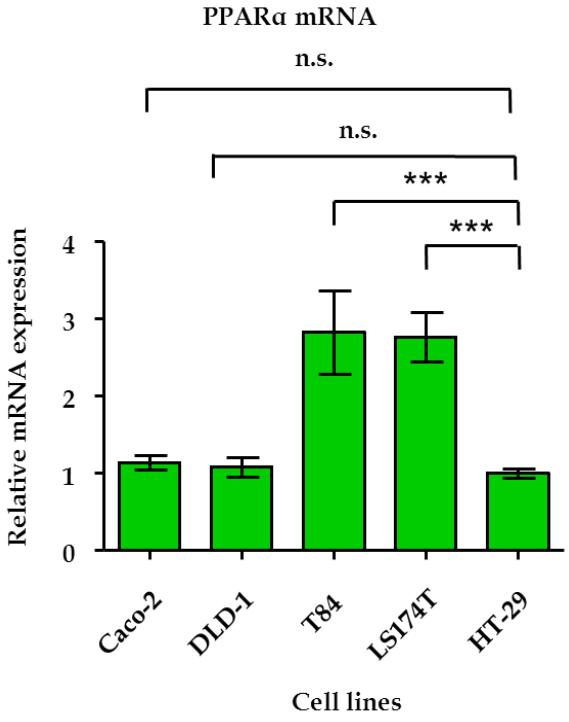


Fig. 6: Endogenous PEPT1 mRNA expression in human cell lines. The PEPT1 mRNA expression levels of Caco-2, DLD-1, T84 and LS174T cells are shown relative to the PEPT1 mRNA of HT-29. The PEPT1 mRNA expression levels were normalized for beta-actin.

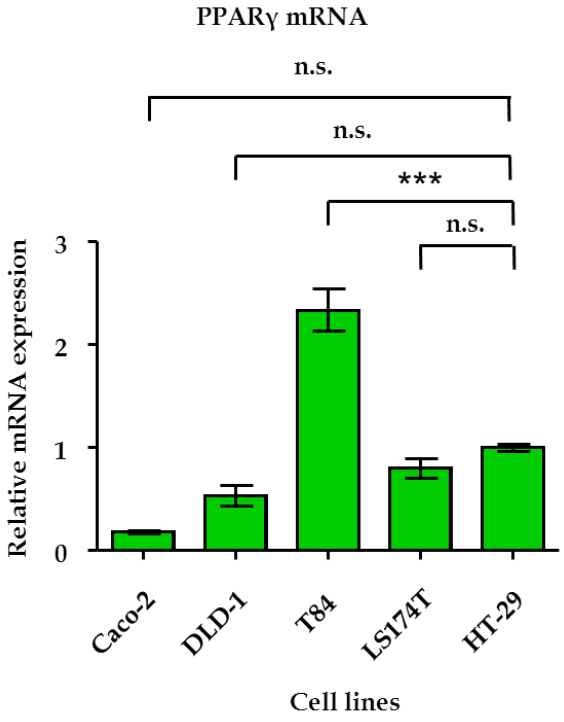
7.1.6 Relative PPARα, PPARγ and RXRα mRNA quantification with real-time PCR in human cell lines

The applicability of the 5 cell lines to study the interaction between PEPT1, PPARα, PPARγ and RXRα was further examined by measuring the relative mRNA levels of the three transcription factors with real-time PCR. The expression level in HT-29 cells was set to 1 and all other cell lines were compared relative to this. The cycle thresholds of PPARα, PPARγ and RXRα of all cell lines were normalized to the cycle thresholds of the housekeeping gene beta-actin. The highest levels of PPARα were found in T84 and LS174T cells (Fig. 7A). Caco-2 and DLD-1 cells had similar expression levels as HT-29. PPARγ had significantly higher mRNA levels in T84 cells. All other cell lines had lower mRNA levels than HT-29 (Fig. 7B). RXRα was similar or lower expressed in all cell lines compared to HT-29 (Fig. 7C).

A



B



C

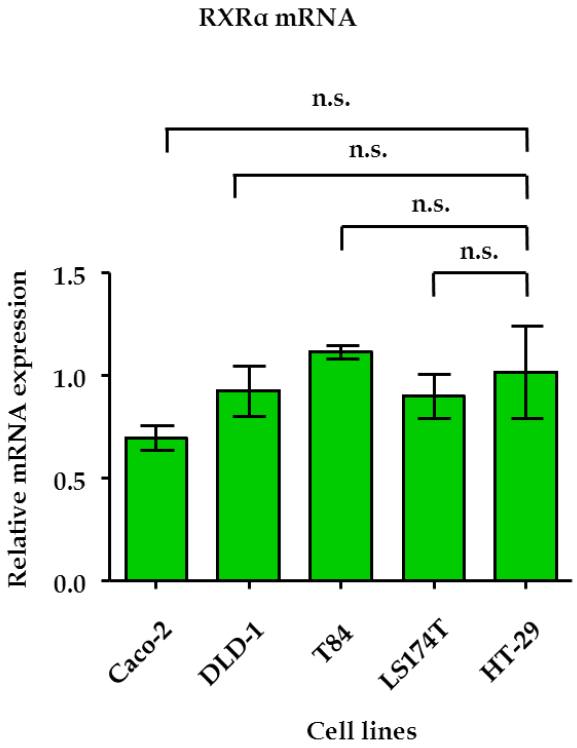


Fig. 7 A-C: Endogenous PPARα, PPARγ and RXRα mRNA expression in human cell lines. The mRNA expression level of a gene of interest was always set in relation to that obtained for HT-29. The mRNA expression levels were normalized to the beta-actin mRNA expression levels of the probes. ***, $p < 0.001$, n.s., not significant.

7.1.7 Relative PEPT1 mRNA quantification with real-time PCR in Caco-2 cells upon treatment with ligands for 48 hours in normal growth medium

The ability of the protein heterodimer PPARγ:RXRα to activate the PEPT1 promoter within living cells was examined by adding different ligands to the Caco-2 growth medium. 9-cis-RA, a natural RXRα agonist, the endogenous agonist 15d-PGJ2 of PPARγ and a synthetic antagonist of PPARγ, GW-9662, were added to the growth medium of Caco-2 cells. After a 48-hour incubation period, the relative mRNA levels of PEPT1 were measured with real-time PCR. The Ct of PEPT1 was normalized to the Ct of the housekeeping gene beta-actin.The addition of either 9-cis-RA, 15d-PGJ2 and

GW-9662 or the combination of 15d-PGJ2/GW9662 resulted in no elevated PEPT1 mRNA expression compared to the control cells only treated with the solvent of the ligands. A slightly lower expression level was observed when the cells were treated with 15d-PGJ2 combined with 9-cis-RA (Fig. 8).

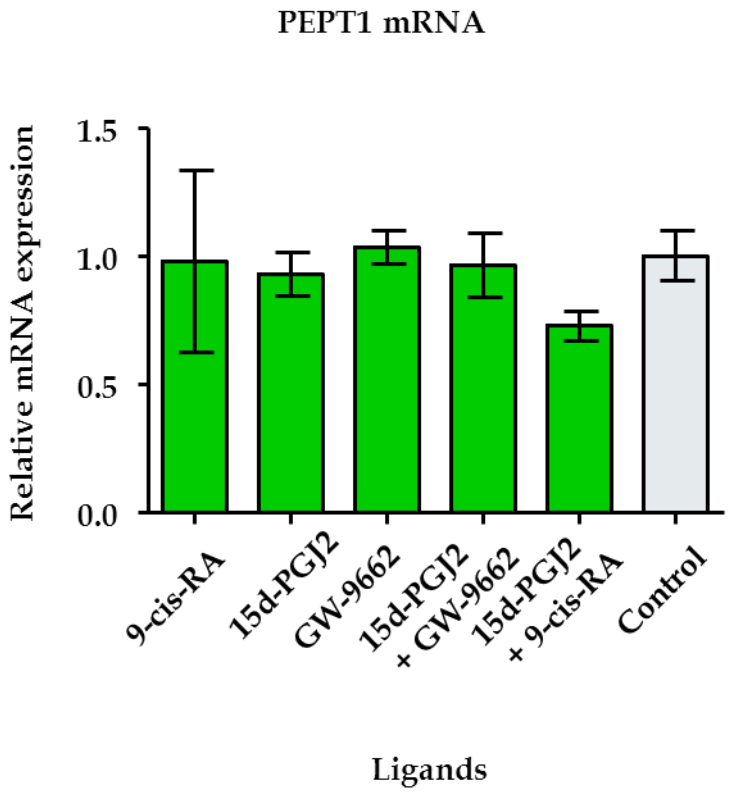


Fig. 8: PEPT1 mRNA expression of Caco-2 cells after 48 hours treatment with different ligands of PPARγ and RXRα in the growth medium. The natural ligands of PPARγ and RXRα, P15d-PGJ2 and 9-cis-RA and the synthetic antagonist of PPARγ, GW-9662, were added either separately or in combination. There were no significant alterations in the PEPT1 mRNA expression compared to the control.

7.1.8 Relative PEPT1 mRNA quantification with real-time PCR in Caco-2 cells treated with ligands for 48 hours in delipidated growth medium

Fetal bovine serum which is added to the growth medium of most cell lines contains free fatty acids. These are natural agonists of PPARγ. To avoid the

interference of the added ligands with the free fatty acids of the growth medium, experiments were conducted with delipidated fetal calf serum. The same ligands, incubation period and normalization procedure as used for the previous experiment were applied to investigate any effect on mRNA levels of PEPT1. The combined addition of 15d-PGJ2 and 9-cis-RA resulted in lower PEPT1 mRNA expression level that showed low significance. The other ligands did not change the mRNA levels compared to the control (Fig. 9).

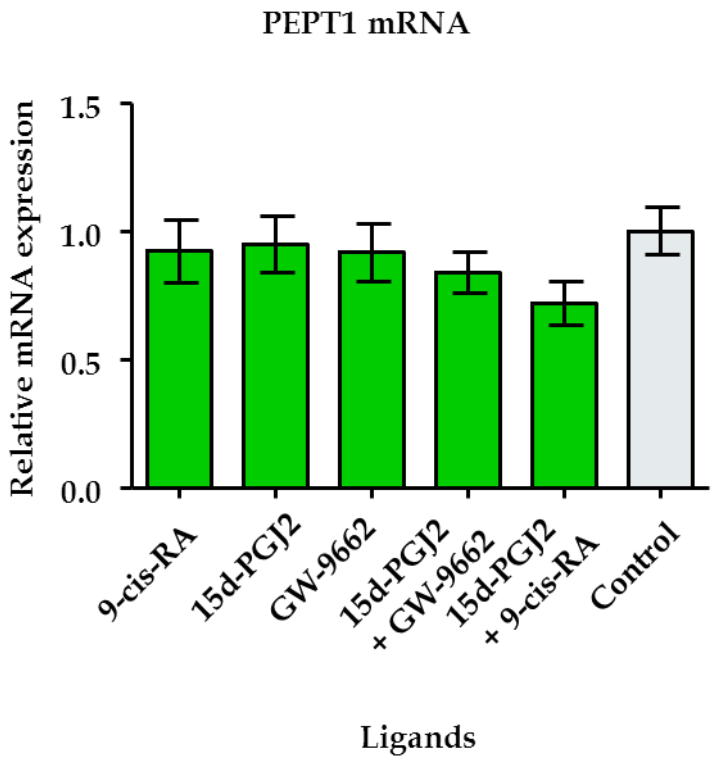


Fig. 9: PEPT1 mRNA expression of Caco-2 cells after 48 hours treatment with different ligands of PPARγ and RXRα in delipidated growth medium. The natural ligands of PPARγ and RXRα, 15d-PGJ2 and 9-cis-RA and the synthetic antagonist of PPARγ, GW-9662, were added either separately or in combination.

7.1.9 Relative PEPT1 mRNA quantification with real-time PCR in Caco-2 cells treated for 24 hours in normal growth medium

The same ligands as in the previous experiments were used with a 24-hours incubation period to investigate potentially altered PEPT1 mRNA levels in Caco-2 cells. The expression levels of PEPT1 mRNA were normalized to those of beta-actin.The addition of 15d-PGJ2 and 15d-PGJ2/GW-9662 resulted in higher but not significant PEPT1 mRNA levels. 9-cis-RA, GW-9662 and PGJ2/9-cis-RA did not increase the expression of PEPT1 mRNA (Fig. 10).

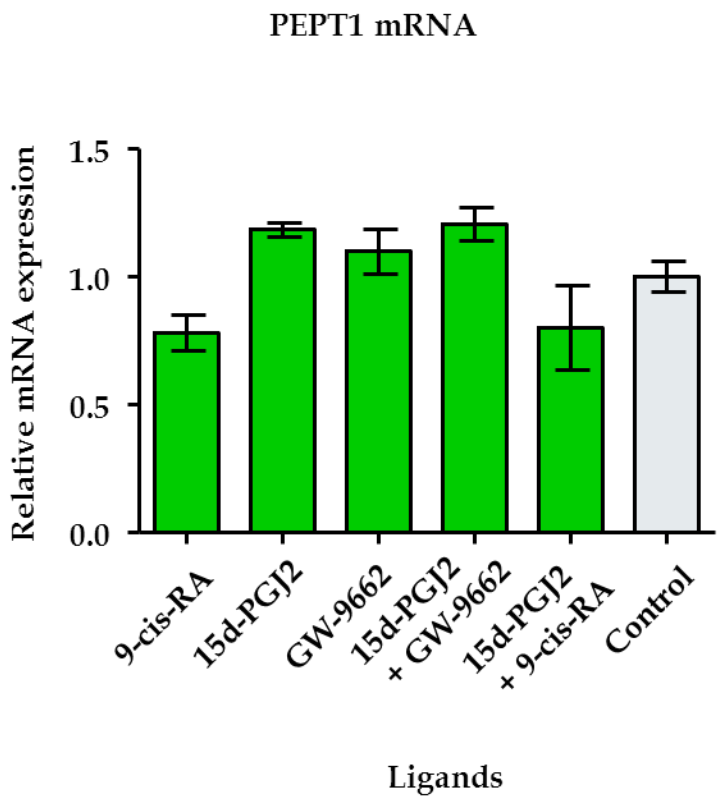


Fig. 10: PEPT1 mRNA expression in Caco-2 cells after 24 hours treatment with different ligands of transcription factors in normal growth medium. The natural ligands of PPARγ and RXRα, 15d-PGJ2 and 9-cis-RA, and the synthetic antagonist of PPARγ, GW-9662, were added either separately or in combination.

7.1.10 Relative PEPT1 mRNA quantification with real-time PCR in Caco-2 cells treated for 24 hours in delipidated growth medium

The same experimental design as described in Figure 10 was also carried out with delipidated growth medium and a 24 hours incubation period. GW-9662 increased the PEPT1 mRNA expression significantly, whereas the other ligands had no significant effect on the endogenous PEPT1 mRNA expression (Fig. 11).

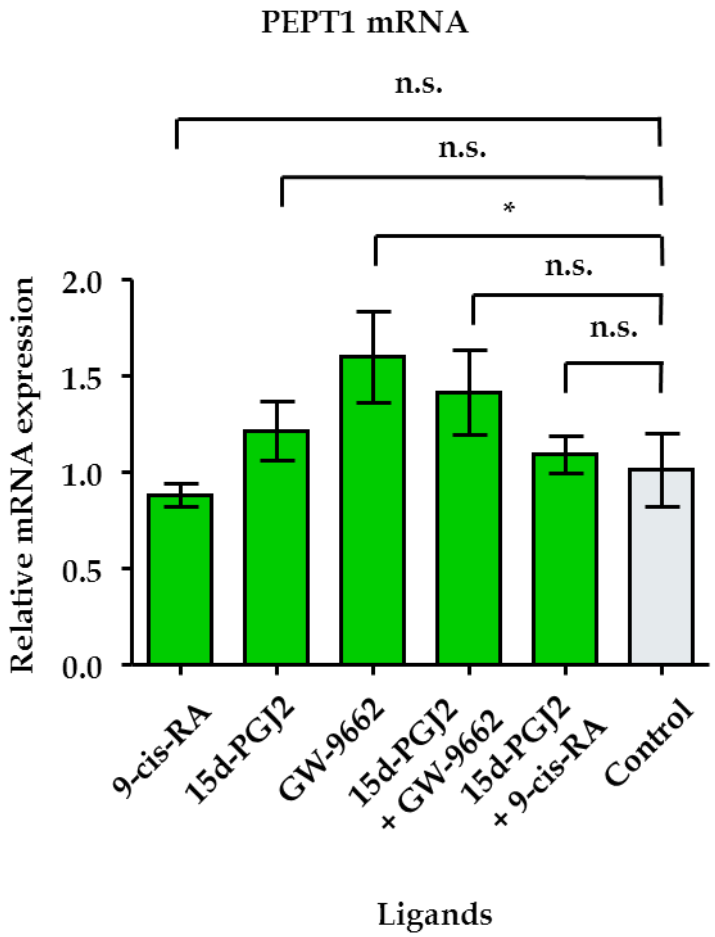
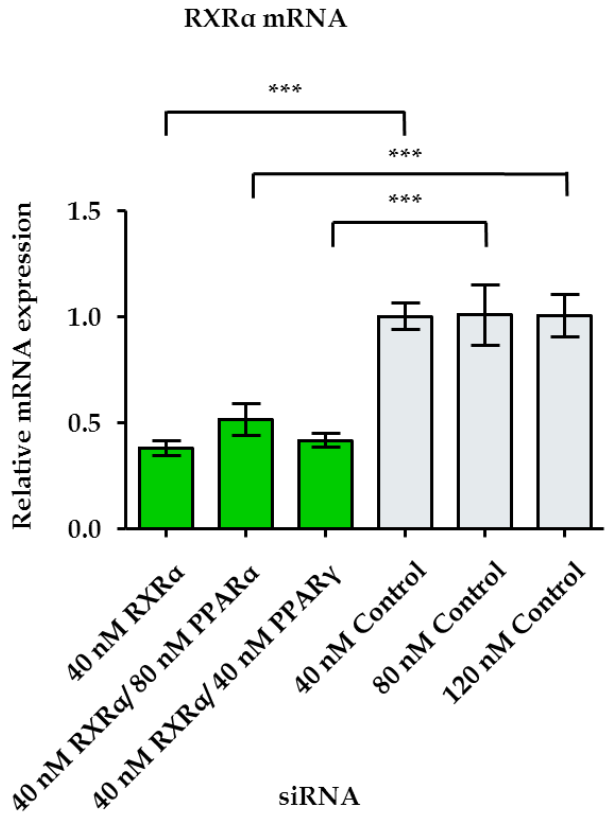


Fig. 11: PEPT1 mRNA expression in Caco-2 cells after 24 hours treatment with different ligands of transcription factors in delipidated growth medium. *, $p < 0.1$, n.s., not significant.

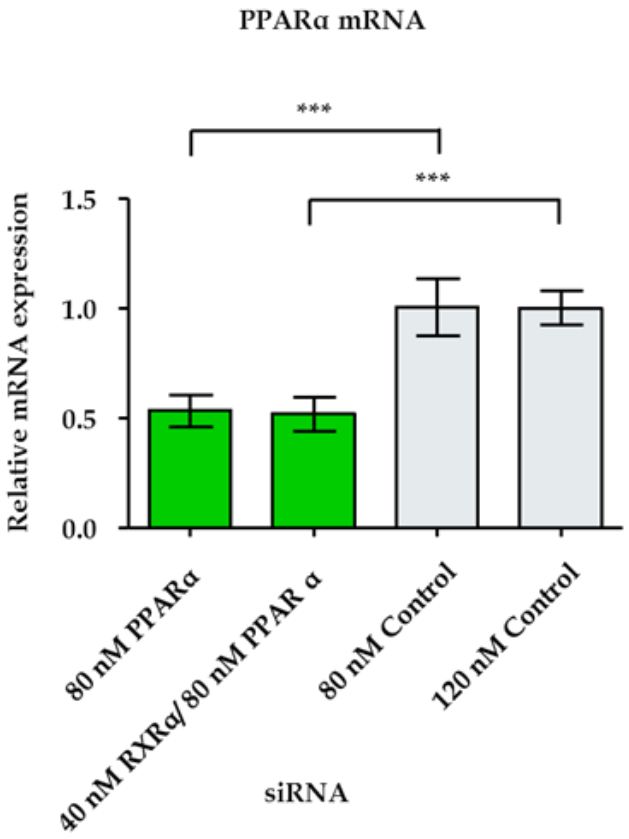
7.1.11 Relative RXRα, PPARα and PPARγ mRNA quantification with real-time PCR in Caco-2 cells treated with siRNA inhibiting the expression of RXRα, PPARα and PPARγ

Knockdown experiments were performed to investigate whether endogenous human PEPT1 mRNA levels are regulated by PPARα, PPARγ or RXRα. The human colon cancer cell line Caco-2 was chosen for this purpose because it expresses PPARα, PPARγ and RXRα, all at detectable levels. The cells were treated over 48 hours with pools of siRNAs specifically targeting one or two transcription factors. For the control groups, non-targeting siRNA pools were used. The mRNA levels of RXRα, PPARα and PPARγ were measured by real time PCR and the results were normalized to beta-actin. Upon RXRα, RXRα/PPARα or RXRα/PPARγ siRNA treatment, there was a significant loss of endogenous RXRα mRNA (Fig. 12 A). Both PPARα and PPARγ mRNA levels were significantly reduced upon the siRNA treatment (Fig. 12 B and C).

A



B


PPARα mRNA
***
***
Relative mRNA expression
1.5
1.0
0.5
0.0
80 nM PPARα
40 nM RXRα/ 80 nM PPAR α
80 nM Control
120 nM Control
siRNA

C

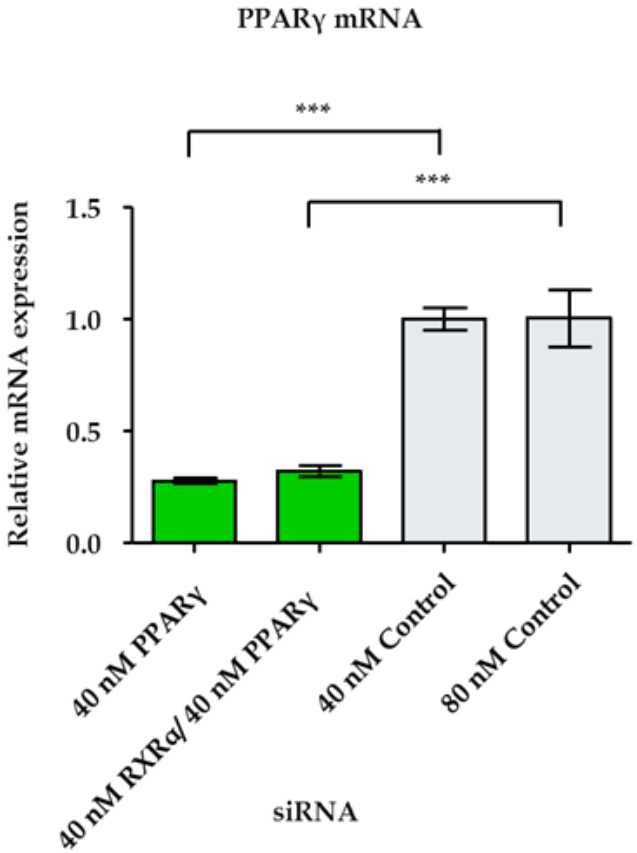

PPARγ mRNA
***
***
Relative mRNA expression
1.5
1.0
0.5
0.0
40 nM PPARγ
40 nM RXRα/ 40 nM PPARγ
40 nM Control
80 nM Control
siRNA

Fig. 12 A-C: Relative RXRα, PPARα and PPARγ mRNA quantification with real-time PCR in Caco-2 cells treated with siRNA inhibiting the expression of RXRα, PPARα and PPARγ. ***, $p < 0.001$.

7.1.12 Relative ASBT mRNA quantification with real-time PCR in Caco-2 cells treated with siRNA inhibiting the expression of RXRα, PPARα and PPARγ

To assess the consequence of markedly reduced levels of RXRα, PPARα and PPARγ mRNA expression, ASBT mRNA levels serving as the positive control were detected by real-time PCR and normalized to beta-actin. Upon PPAR**γ**, PPARα/RXRα and PPARγ/RXRα siRNA treatment, the ASBT mRNA expression was significantly reduced (Jung et al., 2002). Both RXRα and PPARα alone did not alter the ASBT mRNA expression (Fig. 13).

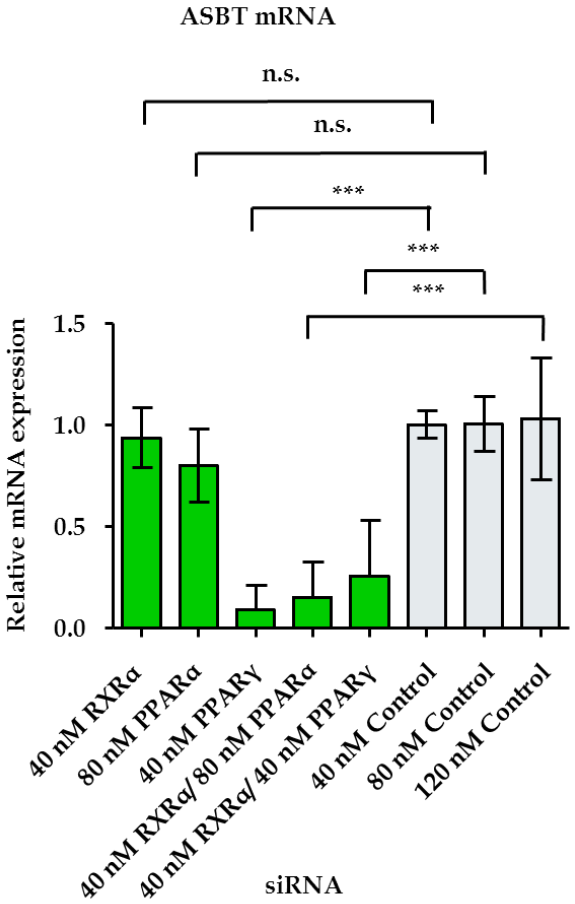


Fig. 13: ASBT mRNA quantification with real-time PCR in Caco-2 cells treated with siRNA inhibiting the expression of RXRα, PPARα and PPARγ. ***, $p < 0.001$, n.s., not significant.

7.1.13 Relative PEPT1 mRNA quantification with real-time PCR in Caco-2 cells treated with siRNA inhibiting the expression of RXRα, PPARα and PPARγ

After verification that the treatment of Caco-2 cells with siRNA targeting RXRα and PPARα expressions reduces the mRNA levels of ASBT (Figure 13), the mRNA levels of PEPT1 were determined and normalized to beta-actin. RXRα, PPARα and PPARγ, alone or in combination, did not alter the expression of PEPT1 mRNA (Fig. 14).

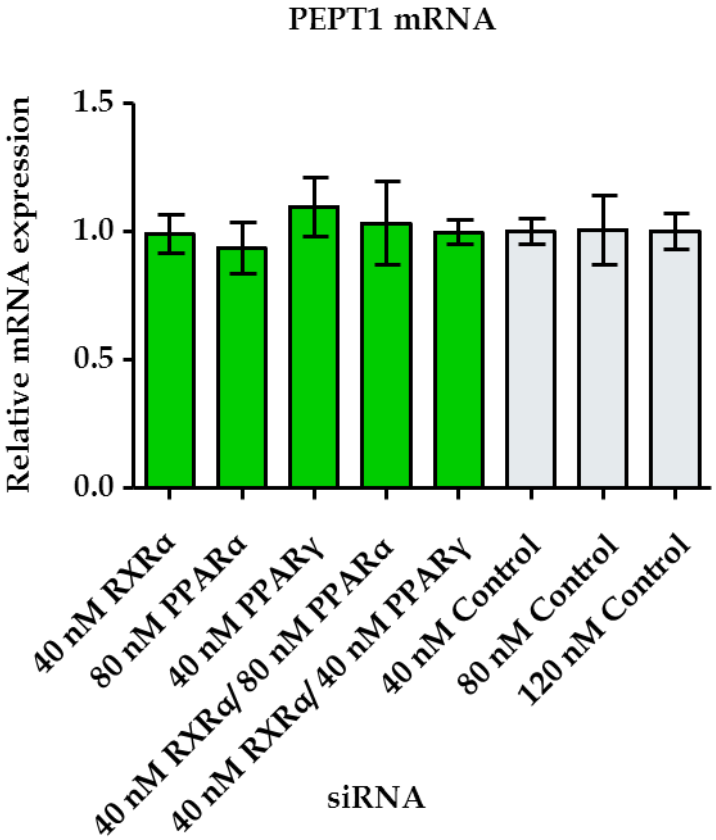


Fig. 14: PEPT1 mRNA quantification with real-time PCR in Caco-2 cells treated with siRNA inhibiting the expression of RXRα, PPARα and PPARγ.

7.1.14 Relative HNF4α mRNA quantification with real-time PCR in Caco-2 cells treated with siRNA inhibiting the expression of RXRα, PPARα, PPARγ and HNF4α

The hepatocyte nuclear factor 4α (HNF4α) binds like PPARs to DR-1 like DNA response elements in order to control the transcription of genes. Knockdown experiments with siRNA inhibiting HNF4α as well as RXRα, PPARα and PPARγ were performed to study a potential involvement in the transcriptional regulation of PEPT1. Caco-2 cells were transfected with inhibiting RNA as mentioned above and the measured real-time PCR signals were normalized to beta-actin. The knockdown of the HNF4α mRNA was significantly lower when 80 nM of HNF4α specific siRNA in combination with PPARα/RXRα or PPARγ/RXRα inhibiting RNA were transfected (Fig. 15). It can be assumed the mechanism inhibiting HNF4α mRNA expression by interfering siRNA is concentration dependent.

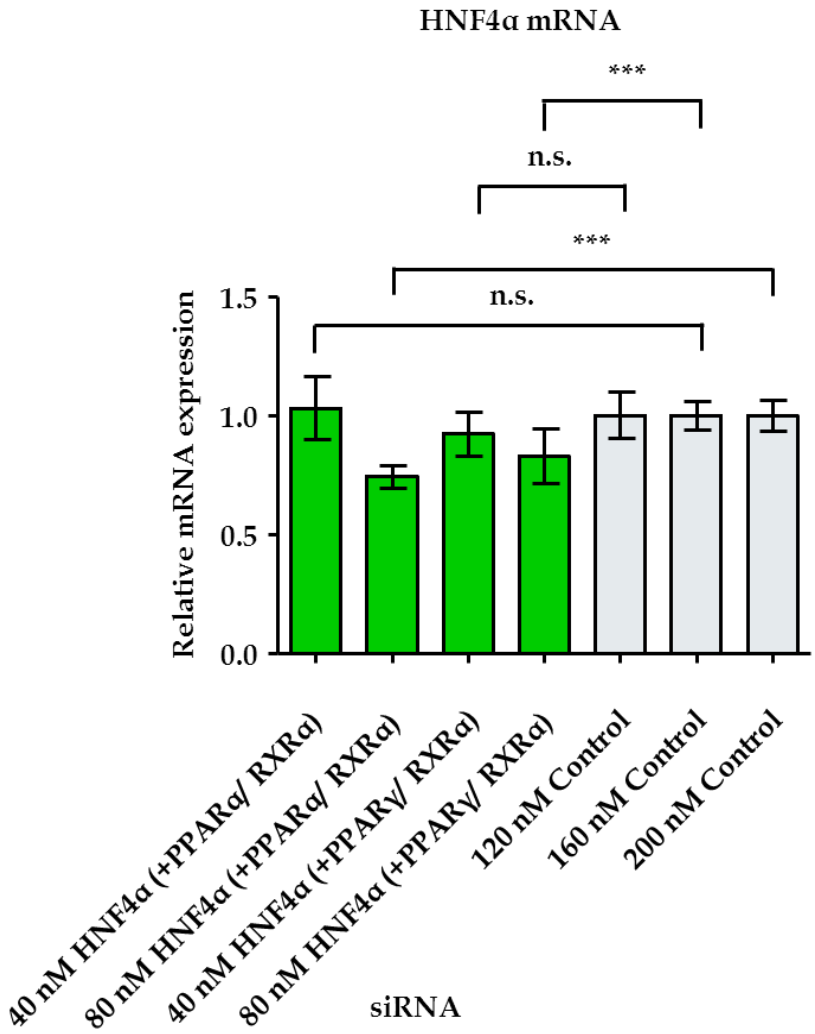
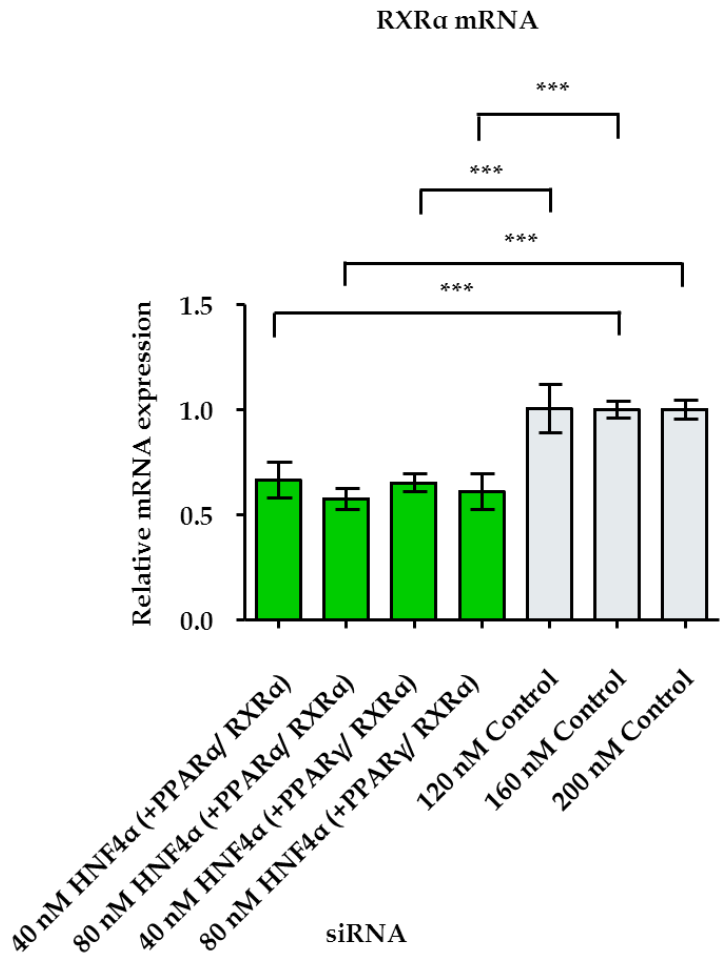


Fig. 15: HNF4α mRNA quantification with real-time PCR in Caco-2 cells treated with siRNA inhibiting the expression of HNF4α, RXRα, PPARα and PPARγ. ***, $p < 0.001$, n.s., not significant.

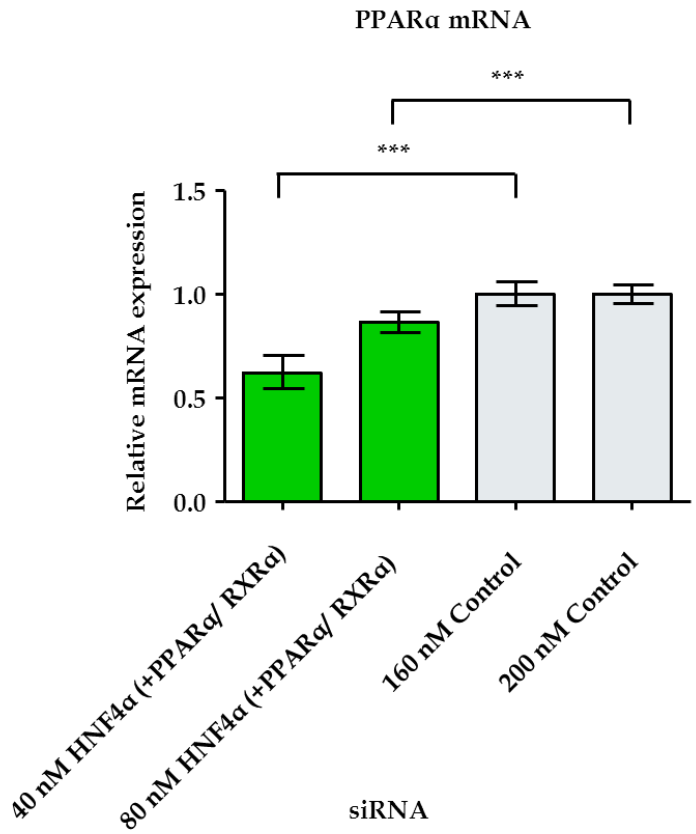
7.1.15 Relative RXRα, PPARα and PPARγ mRNA quantification with real-time PCR in Caco-2 cells treated with siRNA inhibiting the expression of RXRα, PPARα, PPARγ and HNF4α

The knockdown of RXRα, PPARα and PPARγ mRNA was confirmed by real-time PCR and normalizing the results to beta-actin. A significant reduction of the mRNA was observed after treating the cells with the siRNA indicated below (Fig. 16 A-C), except for PPARγ after treating the cells with 40 nM HNF4α siRNA (Fig. 16 C).

A


RXRα mRNA
***
***
***
***
Relative mRNA expression
1.5
1.0
0.5
0.0
40 nM HNF4α (+PPARα/ RXRα)
80 nM HNF4α (+PPARα/ RXRα)
40 nM HNF4α (+PPARγ/ RXRα)
80 nM HNF4α (+PPARγ/ RXRα)
120 nM Control
160 nM Control
200 nM Control
siRNA

B


PPARα mRNA
***
***
Relative mRNA expression
1.5
1.0
0.5
0.0
40 nM HNF4α (+PPARα/ RXRα)
80 nM HNF4α (+PPARα/ RXRα)
160 nM Control
200 nM Control
siRNA

C

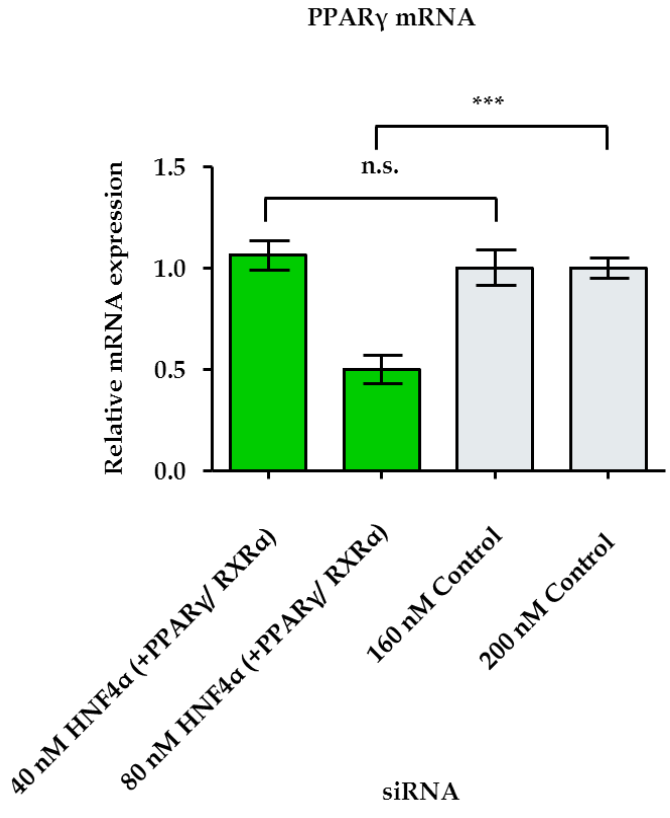

PPARγ mRNA
***
n.s.
Relative mRNA expression
1.5
1.0
0.5
0.0
40 nM HNF4α (+PPARγ/ RXRα)
80 nM HNF4α (+PPARγ/ RXRα)
160 nM Control
200 nM Control
siRNA

Fig. 16 A-C: RXRα, PPARα and PPARγ mRNA quantification with real-time PCR in Caco-2 cells treated with siRNA inhibiting the expression of HNF4α, RXRα, PPARα and PPARγ. ***, $p < 0.001$, n.s., not significant.

7.1.16 Relative PEPT1 mRNA quantification with real-time PCR in Caco-2 cells treated with siRNA inhibiting the expression of RXRα, PPARα, PPARγ and HNF4α

After the verification of the knockdown of the four transcription factors, PEPT1 mRNA levels were measured and normalized to beta-actin. The decrease in the mRNA of RXRα, PPARα, PPARγ and HNF4α did not affect the mRNA levels of PEPT1 (Fig. 17).

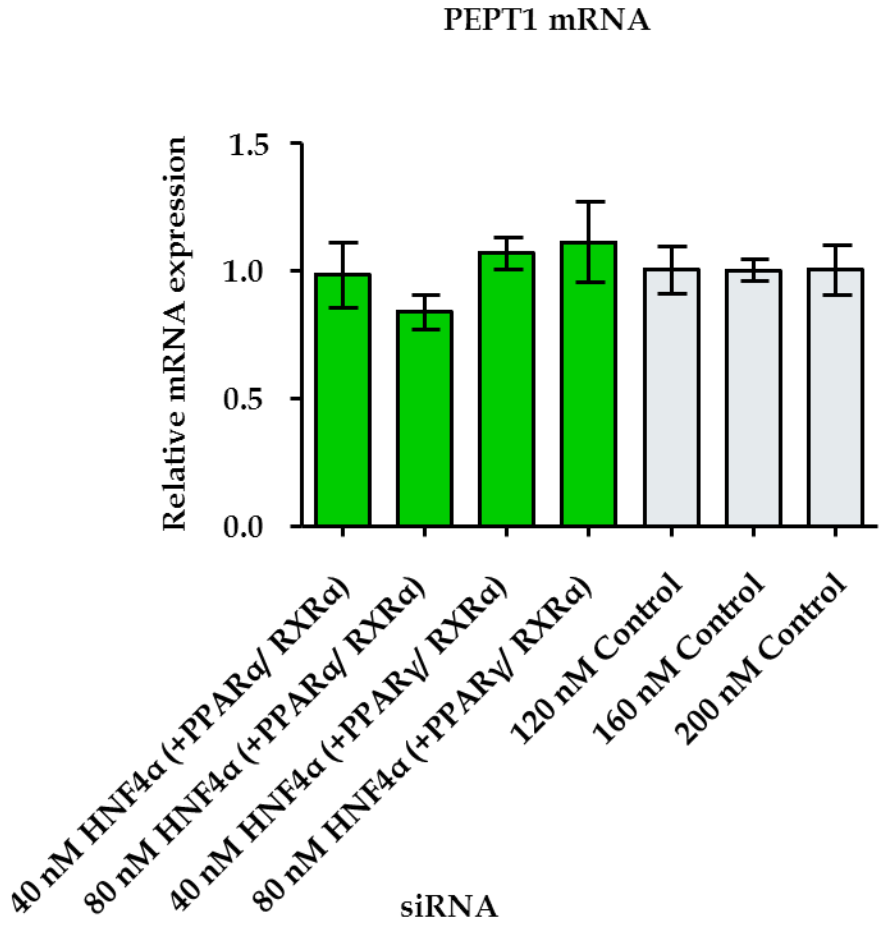


Fig. 17: PEPT1 mRNA quantification with real-time PCR in Caco-2 cells treated with siRNA inhibiting the expression of RXRα, PPARα, PPARγ and HNF4α.

7.1.17 Relative PEPT1 mRNA quantification with real-time PCR in Caco-2 transfected with expression plasmid of PPARγ and RXRα and their respective ligands

Caco-2 cells were co-transfected with expression plasmids bearing the PPARγ or RXRα coding gene 24 hours prior to the treatment with the corresponding ligands. Transfections were performed at a confluency of 70% with 3 µl FuGENE HD/µg DNA. 2 µg of each plasmid were used to transfect cells in Corning 12-well plates. The cells were harvested after 12 – 16 hours incubation in cell culture medium supplemented with 1 µM 15d-PGJ2, 10 µM 9-cis-RA and 1 µM 15d-PGJ2/10 µM 9-cis-RA. The mRNA was relatively quantified and compared to the control cells treated with the vehicle DMSO. 15d-PGJ2 increased the expression of PEPT1 mRNA significantly. The addition of 9-cis-RA alone showed no effect, whereas the combination of both ligands led to the highest relative mRNA expression (Fig. 18).

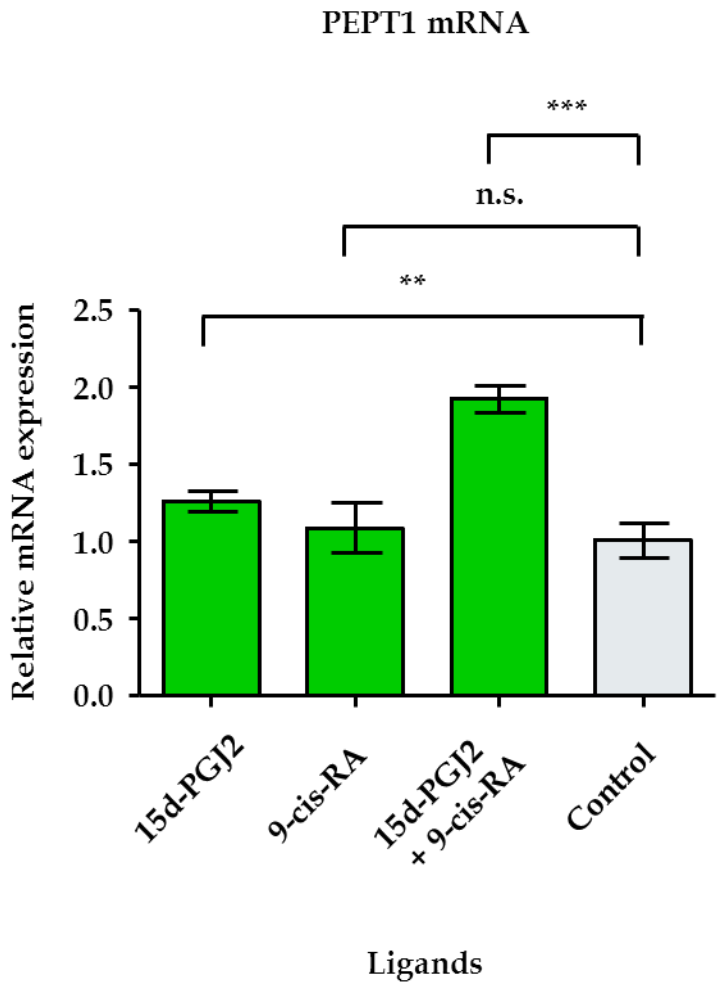


Fig. 18: Caco-2 cells co-transfected with expression plasmids bearing the PPARγ or RXRα coding gene 24 hours prior to the treatment with 1 µM 15d-PGJ2, 10 µM 9-cis-RA and 1 µM 15d-PGJ2/10 µM 9-cis-RA. ***, $p < 0.001$, **, $p < 0.01$, n.s., not significant.

7.1.18 Relative ASBT mRNA quantification with real-time PCR in Caco-2 cells co-transfected with expression plasmid of PPARγ and RXRα and their respective ligands

According to Jung and co-workers it is known that the expression of ASBT is positively regulated by PPARα:RXRα heterodimers (Jung et al., 2002). As the DNA consensus region for PPARα:RXRα is similar for PPARγ:RXRα heterodimers, the relative mRNA levels of ASBT in Caco-2 cells of the previous experiment were

determined as positive control. The combination of 15d-PGJ2 and 9-cis-RA increased the expression of ASBT mRNA significantly. The addition of 15d-PGJ2 or 9-cis-RA alone did not result in a changed expression. (Fig. 19).

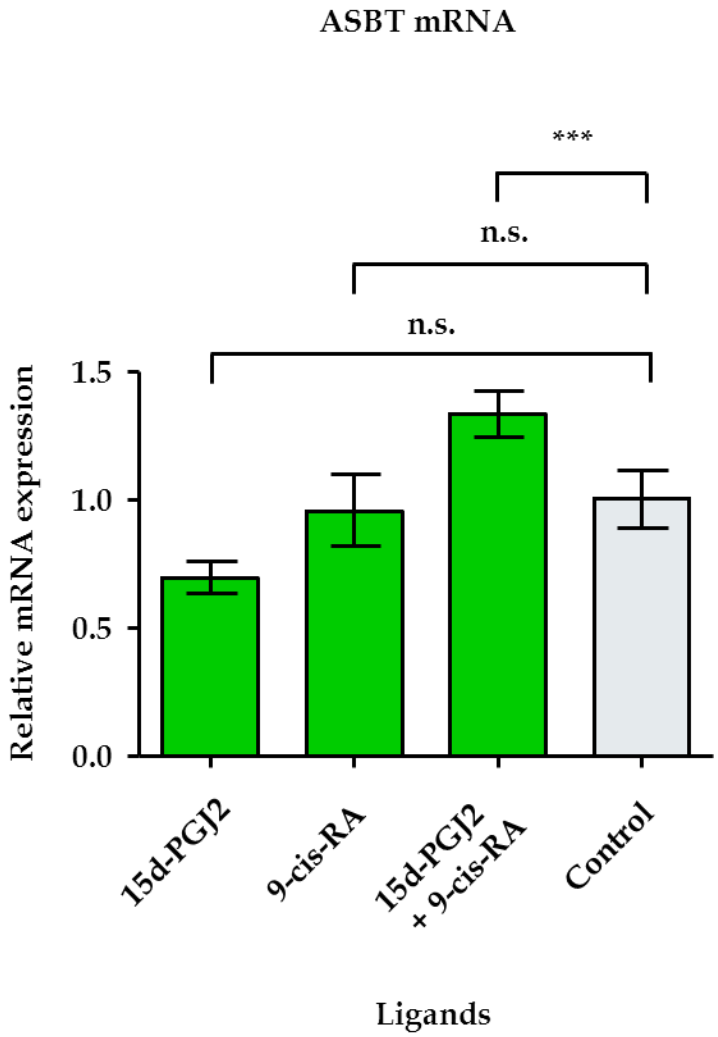


Fig. 19: Relative mRNA levels of ASBT in Caco-2 cells co-transfected with expression plasmids bearing the PPARγ or RXRα coding gene 24 hours prior to the treatment with 1 µM 15d-PGJ2, 10 µM 9-cis-RA and 1 µM 15d-PGJ2/10 µM 9-cis-RA. n.s., not significant, ***, $p < 0.001$.

7.1.19 Relative PEPT1 mRNA quantification with real-time PCR of rat ileal specimens treated with PPARα, PPARγ and RXRα agonists and antagonists

To examine a potential interaction between rat Rxrα, Pparα and Pparγ transcription factors and the rat *Pept1* promoter *ex vivo*, rat ileal specimens were incubated in cell culture medium containing charcoal stripped fetal bovine serum during four hours in a CO_2/RH incubator at 37 °C. Charcoal stripped fetal bovine serum has been absorbed with activated carbon to remove lipophilic material. The reduction of the lipid concentration in the cell medium to a minimum is essential to study properly an activation of PPARs by PGs. 20 µM ciprofibrate and 20 µM WY-14643 were used to activate PPARα, whereas a concentration of 20 µM MK-886 should antagonize the activation. Troglitazone, 15d-PGJ2 and GW-1929 in a final concentration of 20 µM were applied to activate PPARγ. GW-9662 in a concentration of 20 µM served as an inhibitor of PPARγ. The samples were treated either with these ligands, in combinations of these ligands and upon addition of 1 µM 9-cis-RA that is a ligand of RXRα. After four hours incubation period the RNA was isolated, transcribed into cDNA and analyzed by real-time PCR. The addition of either Rxrα, Pparα and Pparγ agonists or antagonists alone or in their respective combinations led to a significant ($p < 0.001$) increase of rPept1 mRNA. The exclusive addition of troglitazone and 15d-PGJ2 showed the smallest increase of rPept1 mRNA ($p < 0.1$) (Fig. 20).

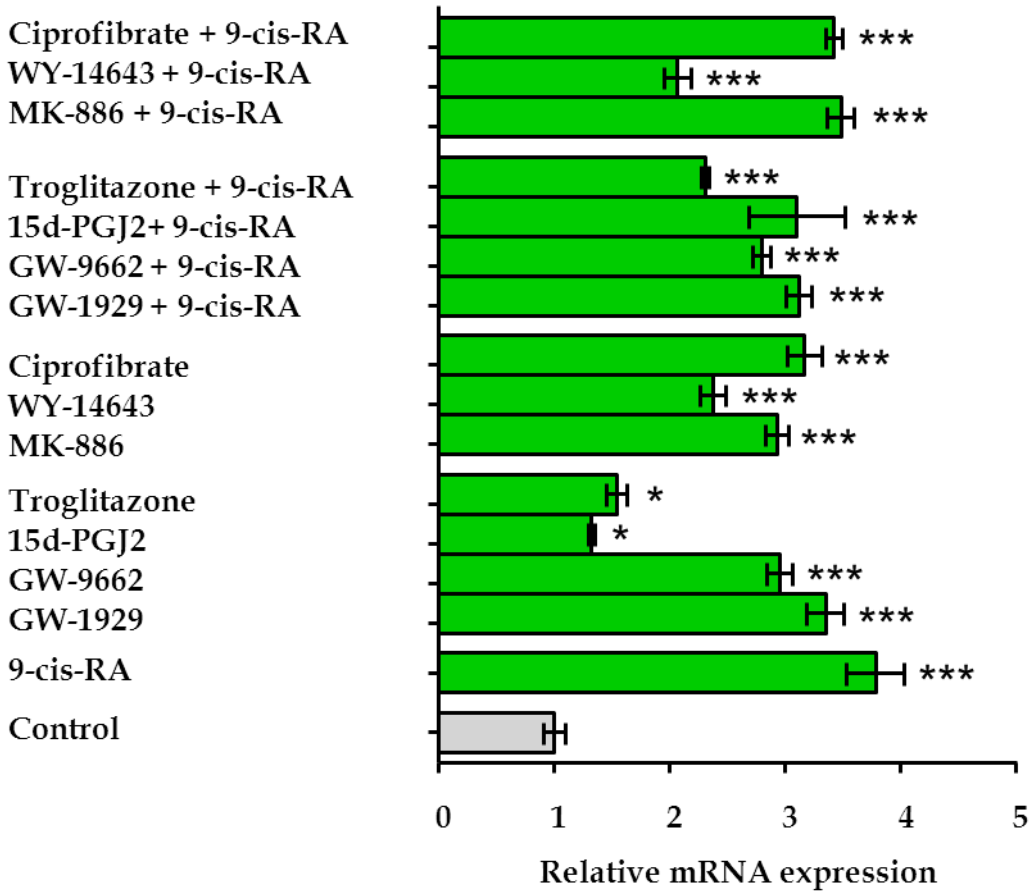


Fig. 20: Relative PEPT1 mRNA quantification with real-time PCR of ileal rat explants treated with PPARα, PPARγ and RXRα agonists and antagonists. 20 µM of all ligands, except 1 µM 9-cis-RA, were used in the treatments. ***, $p < 0.001$, *, $p < 0.1$.

7.1.20 Relative ASBT mRNA quantification with real-time PCR of ileal rat specimens treated with PPARα, PPARγ and RXRα agonists and antagonists

As a positive control the relative mRNA levels of ASBT were determined from the previous experiment (see Fig. 19 for the reference). All ligands added exclusively or in their respective combinations led to an increased relative ASBT mRNA concentration with highest significance ($p < 0.001$). No change was observed upon addition of GW-9662 + 9-cis-RA, WY-14643, troglitazone and 15d-PGJ2 (Fig. 21).

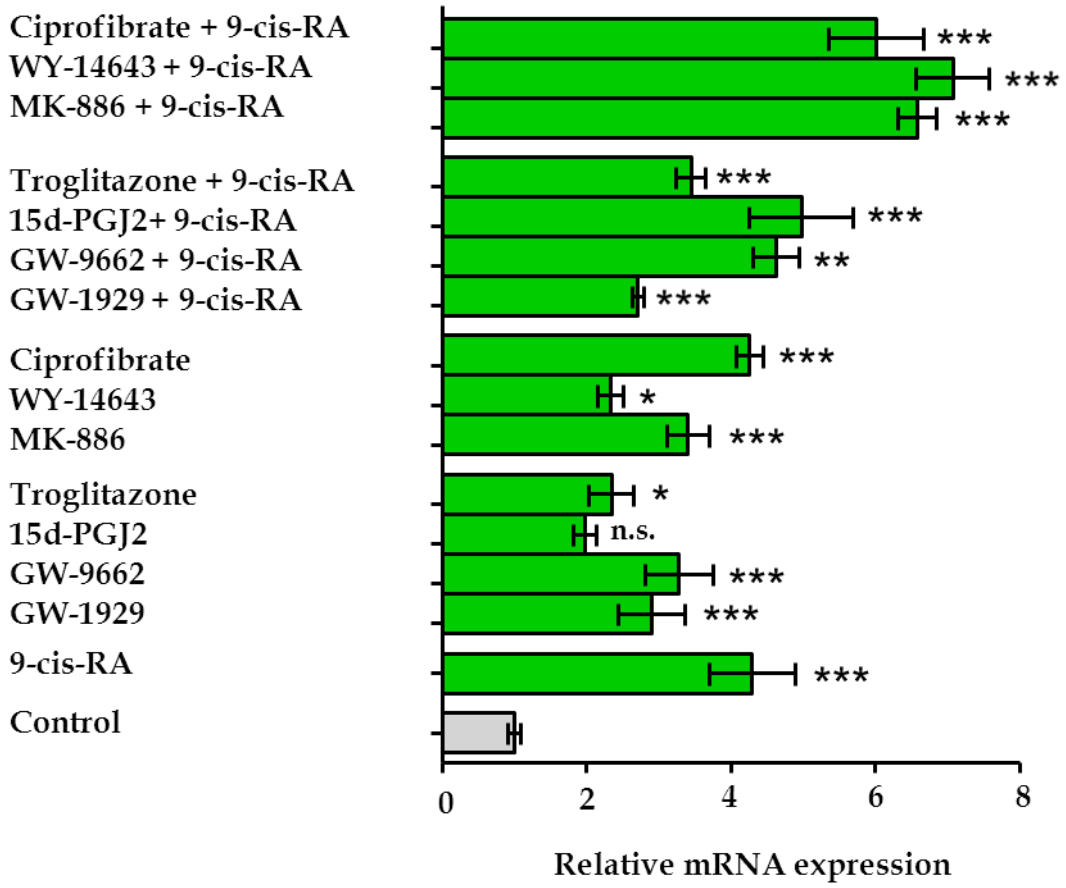


Fig. 21: Relative ASBT mRNA quantification with real-time PCR of ileal rat explants treated with PPARα, PPARγ and RXRα agonists and antagonists. 9-cis-RA was added in a concentration of 1 µM, all the other ligands were 20 µM. ***, $p < 0.001$, **, $p < 0.01$. *, $p < 0.1$. n.s., not significant.

7.2 The organic anion transporting polypeptides (Oatps)

All methodologies applied in this study are listed and described in the section Materials and Methods.

7.2.1 *In silico* identification of putative response elements in mouse *Slco1a1* and *Slco1a4* promoters

Organic anion transporting polypeptides (OATPs/Oatps) are important membrane transport proteins mediating the sodium-independent transport of bile salts, steroid conjugates, thyroid hormones, numerous drugs and xenobiotic compounds. Farnesoid X receptor (Fxr) knockout mice fed with bile acids developed decreased mRNA levels of Oatp1a1 (formerly Oatp1) in the liver compared to wildtype mice (Sinal et al., 2000). In hepatocyte nuclear factor 1α (Hnf1α) and hepatocyte nuclear factor 4α (Hnf4α) knockout mice, Oatp1a1mRNA was down regulated (Shih et al., 2001, Hayhurst et al., 2001). The *Slco1a4* (former Oatp2) gene expression was decreased in Hnf1α knockout mice and increased by activation of the pregnane X receptor (Pxr) (Shih et al., 2001, Staudinger et al., 2001). However, the detailed molecular mechanisms leading to the activation or silencing of *Slco1a1* and *Slco1a4* promoters in rodents remain to be elucidated. To obtain insights into the regulation processes, several constructs of both promoters (NCBI accession number: NW_001030820) were cloned to identify the transcription factor binding sites involved in the promoter regulation using luciferase reporter assays and EMSAs (Fig. 22 and Fig. 23).

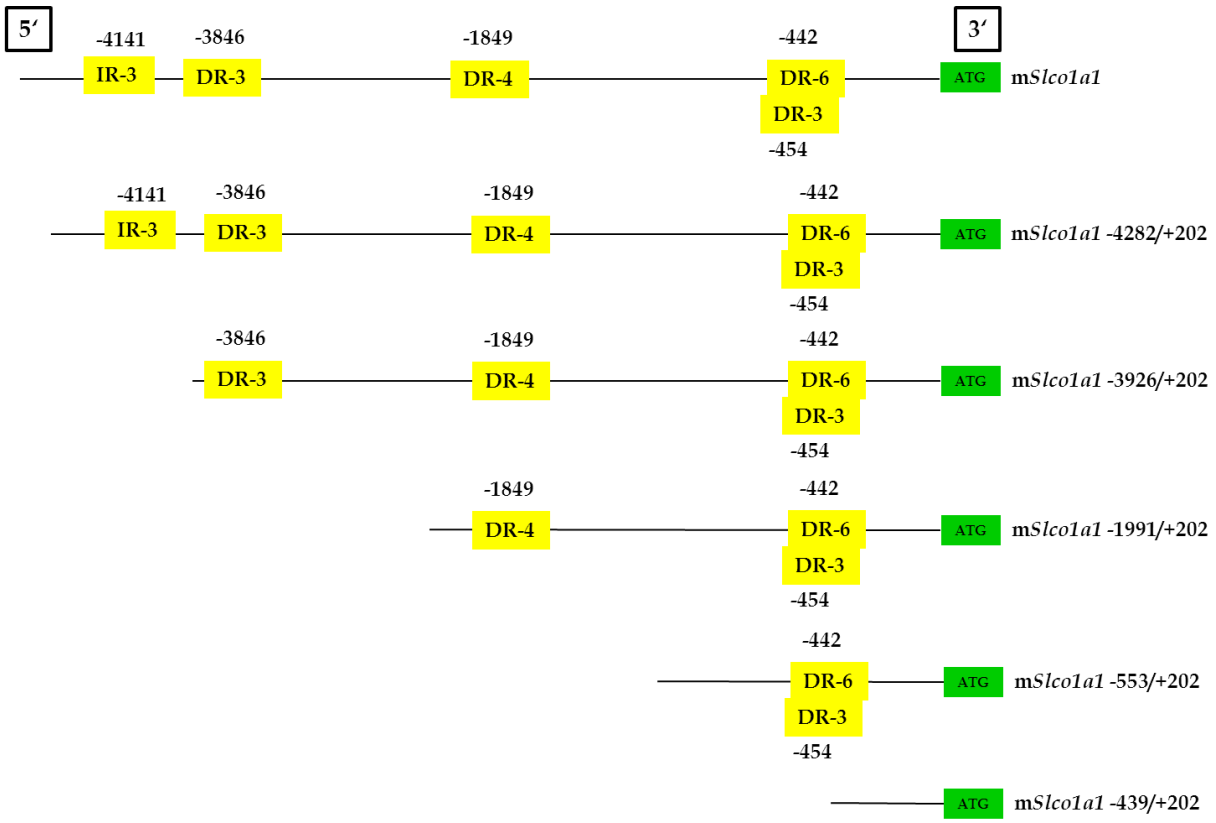


Fig. 22: Mouse *Slco1a1* promoter sequences and constructs. The parts of the promoters cloned into luciferase reporter vectors are indicated by their terminal nucleotides. The 5′-end and the 3′-end of the cloned constructs are indicated with -/+ in relation to the start of transcription. The longest shown promoter sequence represents the template used to clone the constructs. Yellow boxes: putative binding sites of transcription factor, green boxes: start of translation (ATG), IR-3: inverted repeat with a 3 nucleotide spacer, DR-3: direct repeat with a 3 nucleotide spacer, DR-4: direct repeat with a 4 nucleotide spacer, DR-6: direct repeat with a 6 nucleotide spacer.

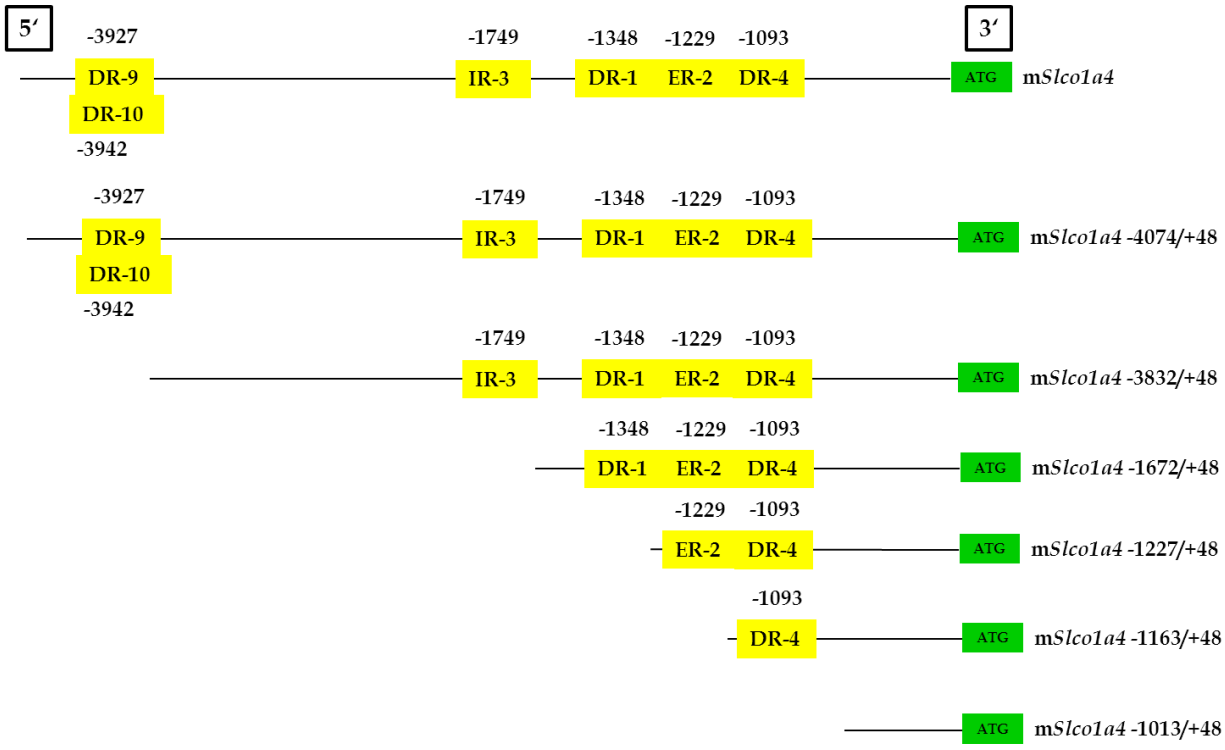


Fig. 23: Mouse *Slco1a4* promoter sequences and constructs. The parts of the promoters cloned into luciferase reporter vectors are indicated by their terminal nucleotides. The 5′-end and the 3′-end of the cloned constructs are indicated with -/+ in relation to the start of transcription. The longest shown promoter sequence represents the template used to clone the constructs. Yellow boxes: binding sites of transcription factors, green boxes: start of translation (ATG), IR-3: inverted repeat with a 3 nucleotide spacer, DR-1: direct repeat with a 1 nucleotide spacer, DR-4: direct repeat with a 4 nucleotide spacer, DR-9: direct repeat with a 9 nucleotide spacer, DR-10: direct repeat with a 10 nucleotide spacer ER-4: everted repeat with a 4 nucleotide spacer.

7.2.2 Transactivation of the mouse *Slco1a1* and *Slco1a4* promoters by the human glucocorticoid receptor (GR)

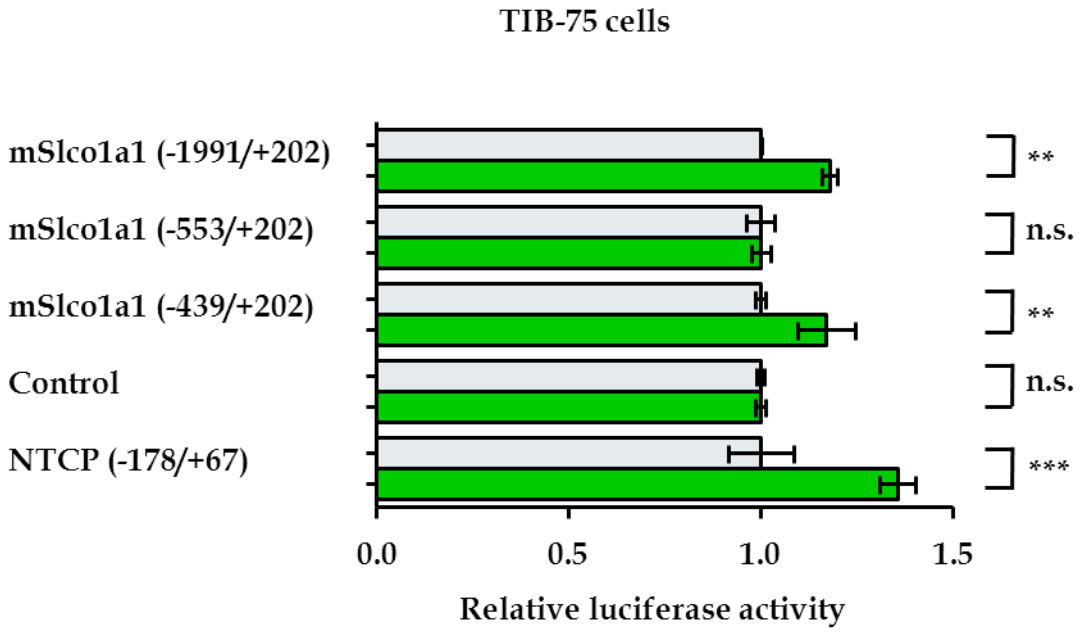
The m*Slco1a1* (-1991/+202), m*Slco1a1* (-553/+202) and m*Slco1a1* (-439/+202) promoters linked to luciferase reporter genes were co-transfected together with the human GR expressing plasmid pSG5-GR into the human liver derived cell line HuH-

7, and the mouse liver cell line TIB-75. A final concentration of 200 nM dexamethasone was used to activate GR. The promoter fragment of the human sodium taurocholate co-transporting polypeptide (*NTCP*) known to be activated by GR in the presence of dexamethasone served as a positive control (Eloranta et al., 2006). In mouse liver derived TIB-75 cells, the m*Slco1a1* (-1991/+202) promoter construct covering the DR-3, DR-4 or DR-6 repeats was 1.2 fold activated. The m*Slco1a1* (-439/+202) promoter fragment which did not contain any predicted response element showed also an increased activity. The promoter was not activated by the DR-3 or DR-6 element present in the m*Slco1a1* (-553/+202) construct. The positive control *NTCP* (-178/+67) showed a 1.4 fold increased activity (Fig. 24 A).

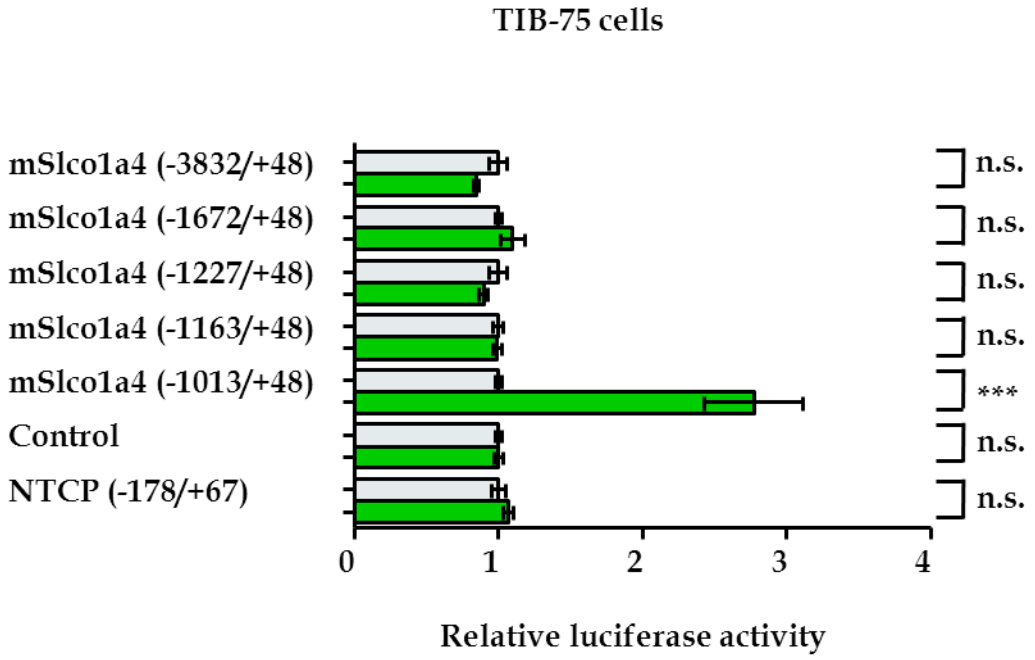
The m*Slco1a4* (-1013/+48) construct was significantly activated whereas the longer promoter fragments and the positive control did not show an increased activity compared to the negative control (Fig. 24 B).

In HuH-7 cells the m*Slco1a1* (-1991/+202) promoter construct had a tendency to higher activity whereas the shorter fragments did not show any significant effects. All m*Slco1a4* constructs were not activated by GR. The positive controls were activated significantly in both experiments (Fig. 24 C and D).

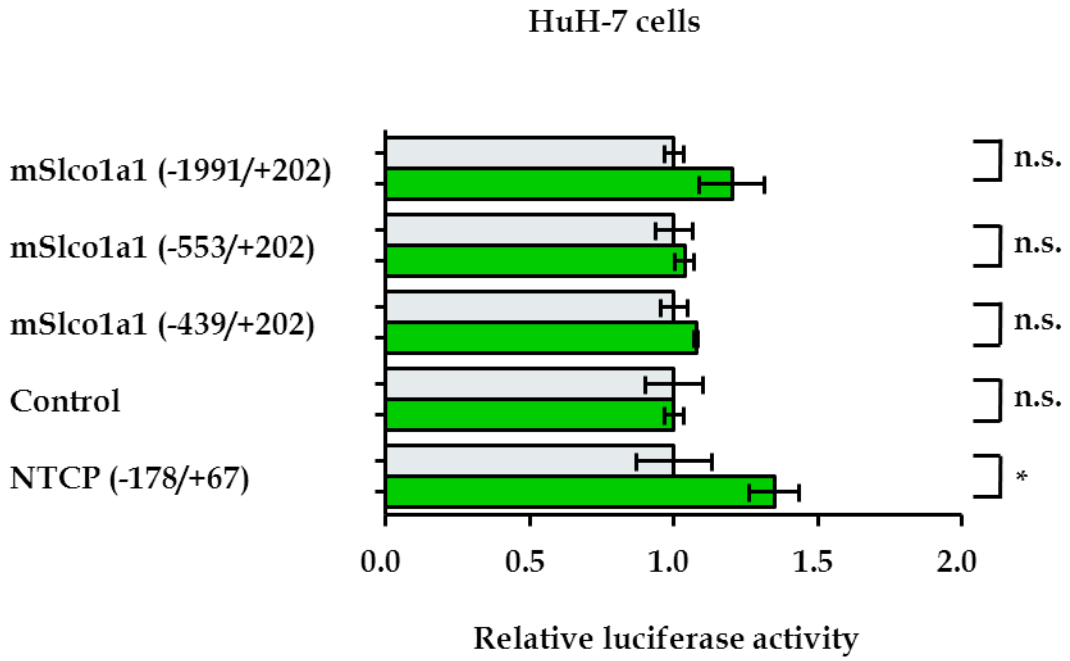
A



B


TIB-75 cells
mSlco1a4 (-3832/+48)
mSlco1a4 (-1672/+48)
mSlco1a4 (-1227/+48)
mSlco1a4 (-1163/+48)
mSlco1a4 (-1013/+48)
Control
NTCP (-178/+67)
n.s.
n.s.
n.s.
n.s.
***
n.s.
n.s.
0
1
2
3
4
Relative luciferase activity

C


HuH-7 cells
mSlco1a1 (-1991/+202)
mSlco1a1 (-553/+202)
mSlco1a1 (-439/+202)
Control
NTCP (-178/+67)
n.s.
n.s.
n.s.
n.s.
*
0.0
0.5
1.0
1.5
2.0
Relative luciferase activity

D

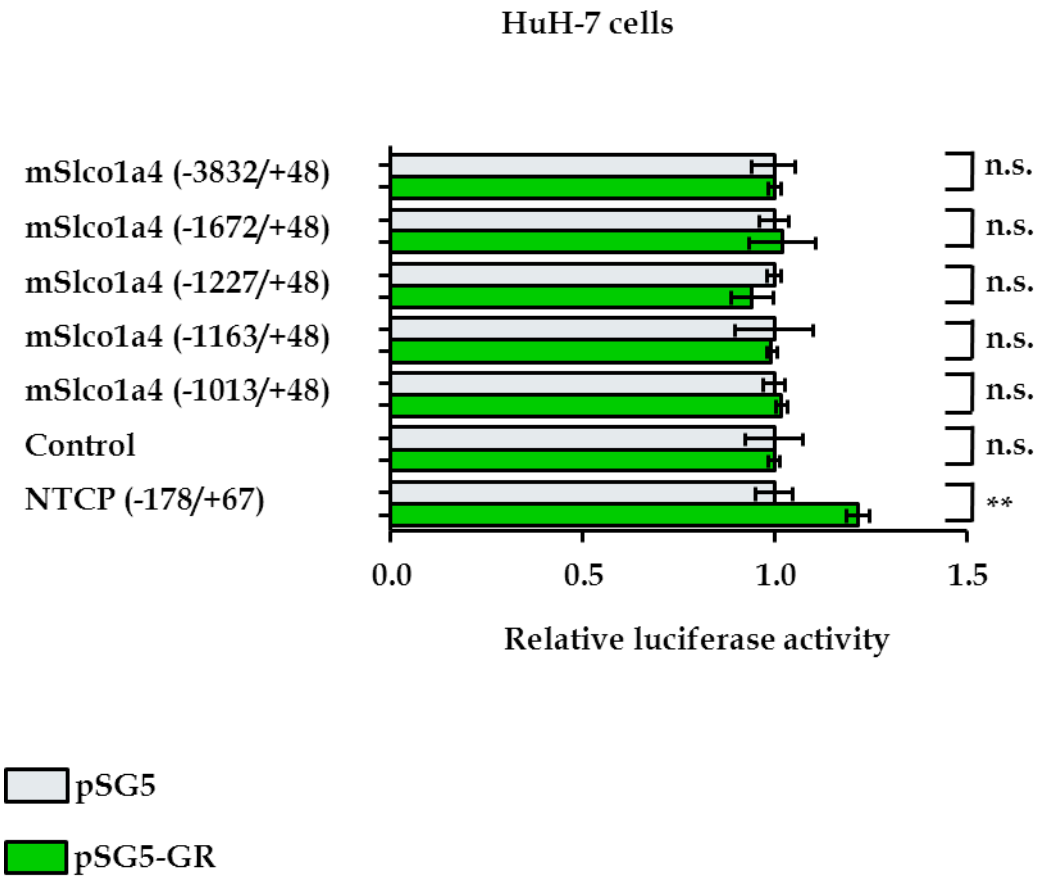


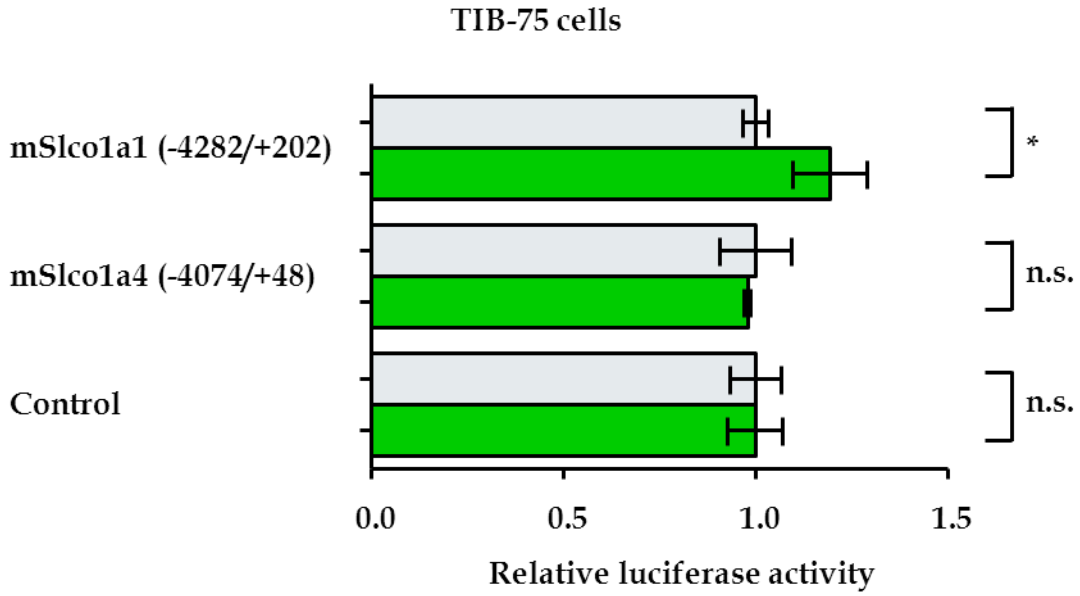
Fig. 24 A-D: Co-transfections of TIB-75 and HuH-7 cells with m*Slco1a1* and m*Slco1a4* promoter constructs and GR expressing plasmids in the presence of 200 nM dexamethasone. ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.1$; n.s., not significant.

7.2.3 Transactivation of the mouse *Slco1a1* and *Slco1a4* promoters by the mouse glucocorticoid receptor (mGr)

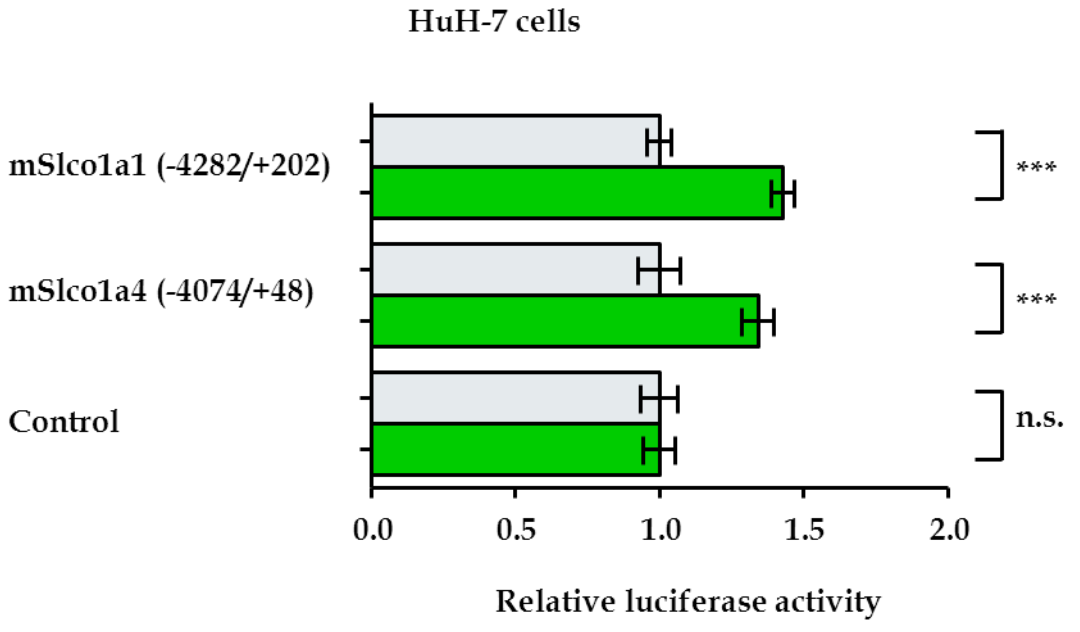
In the experiments described above, the human glucocorticoid receptor was used to examine mouse promoter sequences in a rodent cell line. The mouse glucocorticoid receptor (mGr) was cloned into the expression vector pSG5 to investigate whether mouse specific transcriptional regulation mechanisms would occur. The m*Slco1a1* (-4282/+202) and m*Slco1a4* (-4074/+48) promoters were co-transfected together with pSG5-mGr into TIB-75 and HuH-7 cells. In the presence of the ligand dexamethasone in a final concentration of 200 nM, mGr activated in the

mouse liver cell line the m*Slco1a1* construct 1.2 fold, but not m*Slco1a4* (Fig. 25 A). In HuH-7 cells both constructs were significantly activated (Fig. 25 B).

A



B



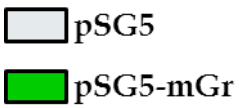
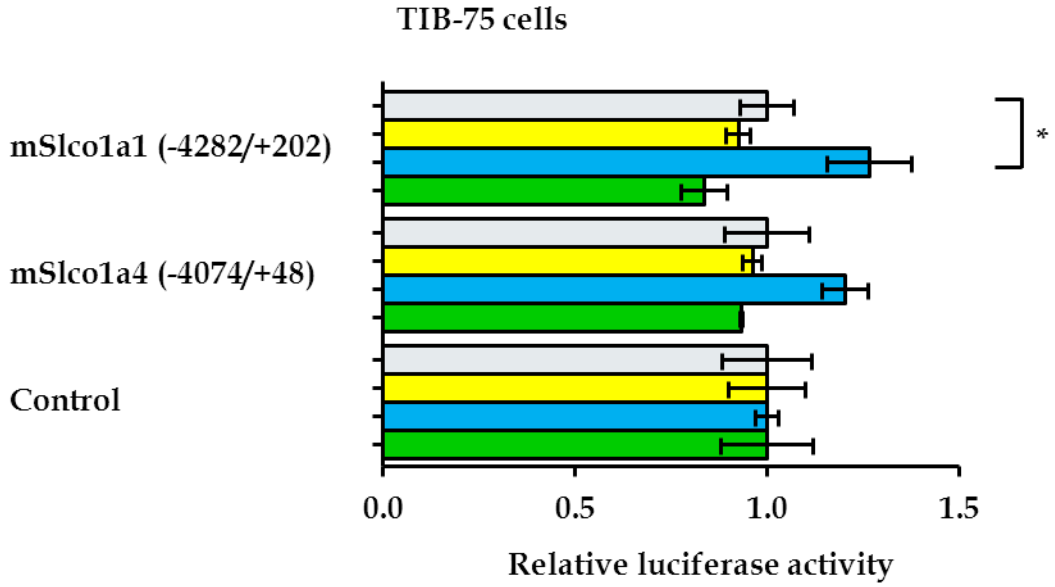


Fig. 25 A and B: Transactivation of the mouse *Slco1a1* and *Slco1a4* promoters by the mouse glucocorticoid receptor (mGr) in TIB-75 and HuH-7 cells in the presence of 200 nM of its ligand dexamethasone. ***, $p < 0.001$; *, $p < 0.1$; n.s., not significant.

7.2.4 Transactivation of the mouse *Slco1a1* and *Slco1a4* promoters by the mouse mPxr:mRxrα heterodimer

The human pregnane X receptor (PXR) interacts with RXRα to form heterodimers and recognizes DR-3, DR-4 or ER-6 elements of promoters (Vyhlidal et al., 2004). The mouse isoforms of PXR and RXRα were employed in the co-transfection assays. Increasing final concentrations from 10 µM to 100 µM of pregnenolone-16α-carbonitrile (PCN), a rodent Pxr activator, were tested. The m*Slco1a1* (-4282/+202) promoter was significantly activated in TIB-75 cells only in the presence of 50 µM PCN (Fig 26 A). In HuH-7 cells the promoter showed increased activity when treated with 10 µM and 100 µM but not with 50 µM PCN (Fig 26 B). The m*Slco1a4* promoter showed a small increase in TIB-75 cells but not in human HuH-7 cells (Fig 26 A and B).

A



B

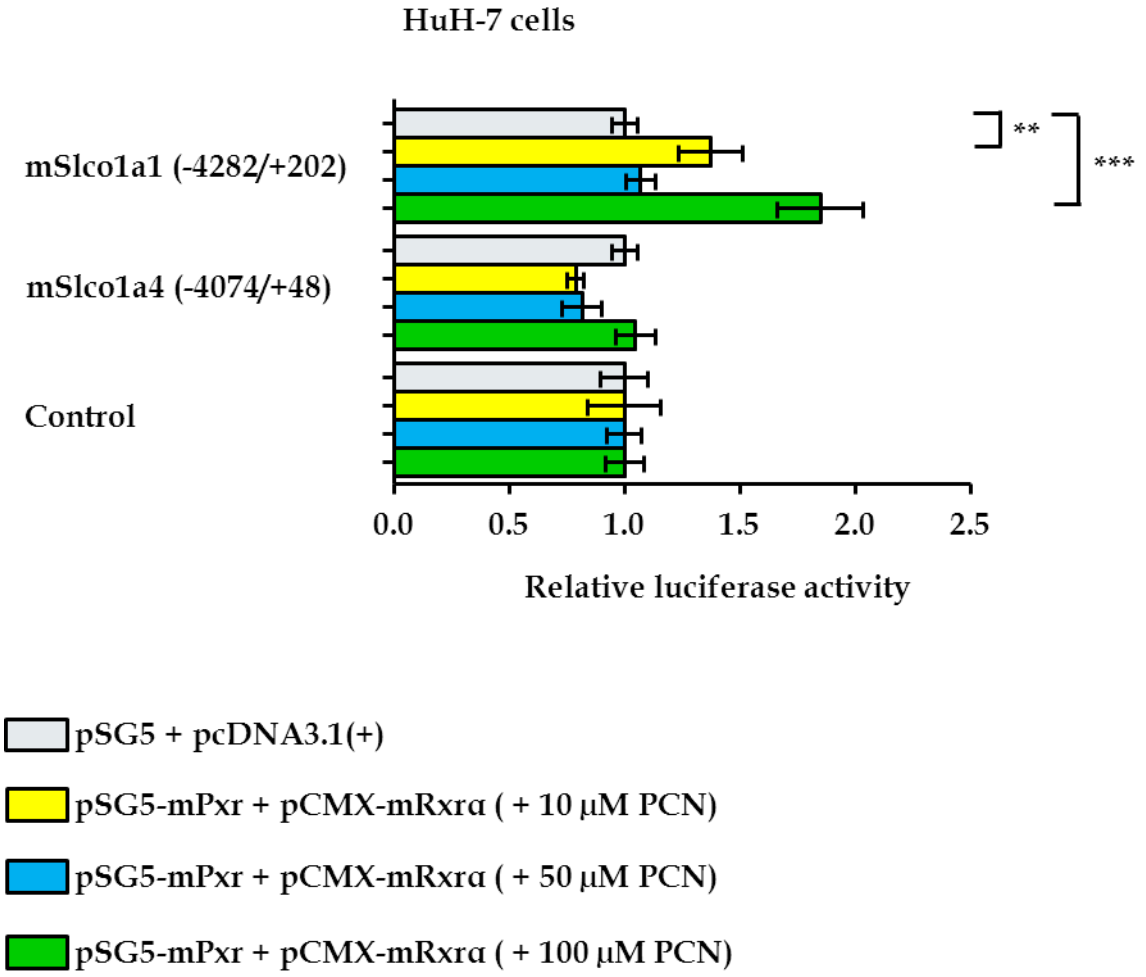


Fig. 26 A and B: Co-transactivation of the mouse *Slco1a1* and *Slco1a*4 promoters by the mPxr:mRxrα heterodimer in TIB-75 and HuH-7 cells. Concentrations of 10 µM, 50 µM and 100 µM of the rodent Pxr activator pregnenolone-16α-carbonitrile (PCN) were tested. ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.1$.

7.2.5 Transactivation of the mouse *Slco1a1* and *Slco1a4* promoters by the mouse mPxr:mRxrα heterodimer and mGr

As a further *in vitro* model system to study the promoter activities, HepG2 cells, a human hepatoblastoma cell line, were used in co-transfection assays. Neither m*Slco1a1* nor m*Slco1a4* was significantly activated by mPxr:mRxrα or mGr in the presence of their respective ligands, 10 µM PCN or 200 nM dexamethasone (Fig. 27).

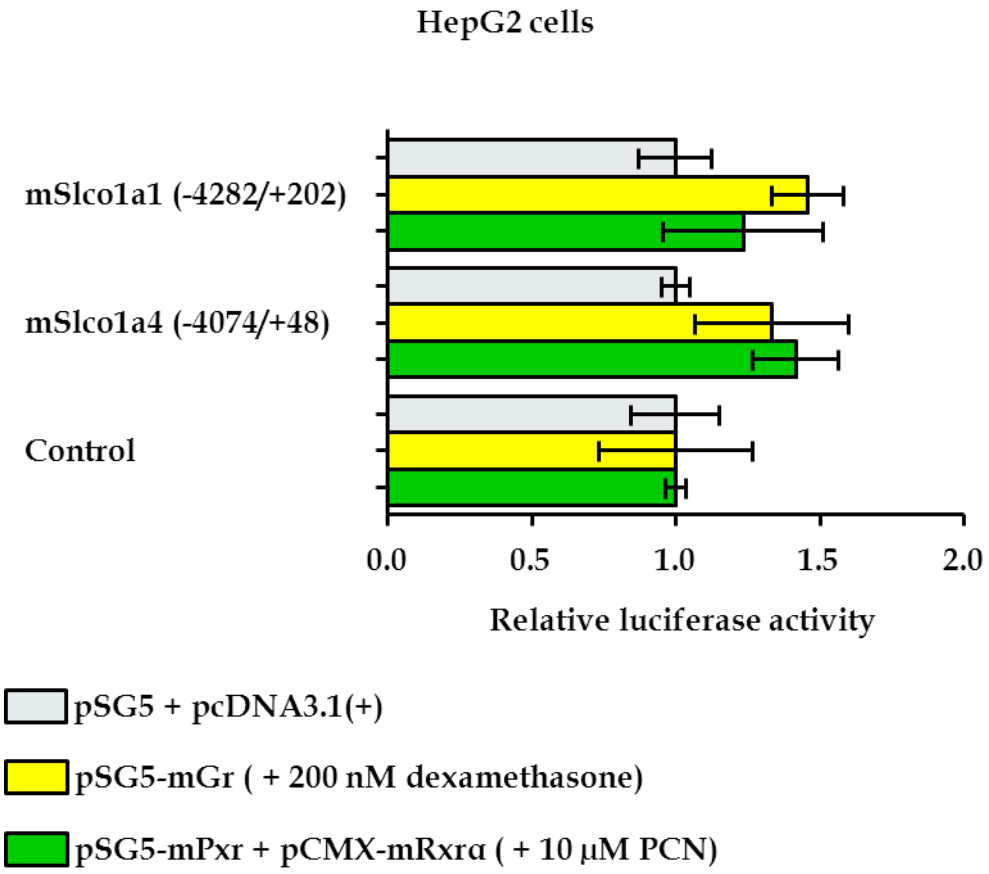


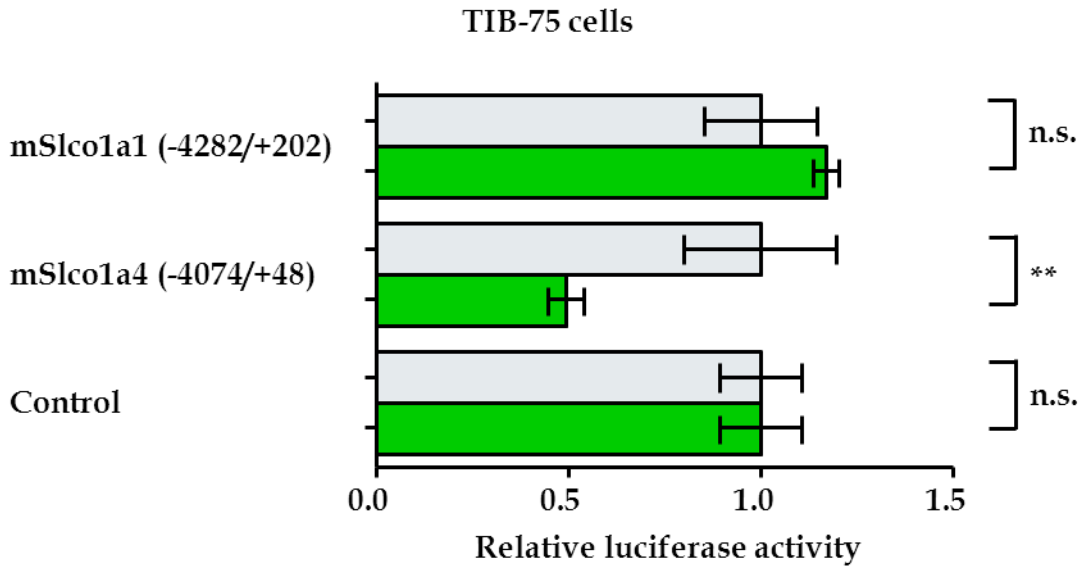
Fig. 27: Co-transactivation of the mouse *Slco1a1* and *Slco1a4* promoters by mPxr:mRxrα heterodimer and mGr. Final concentrations of 10 µM PCN or 200 nM dexamethasone were used to activate mPxr or mGr.

7.2.6 Transactivation of the mouse *Slco1a1* and *Slco1a4* promoters by FXR

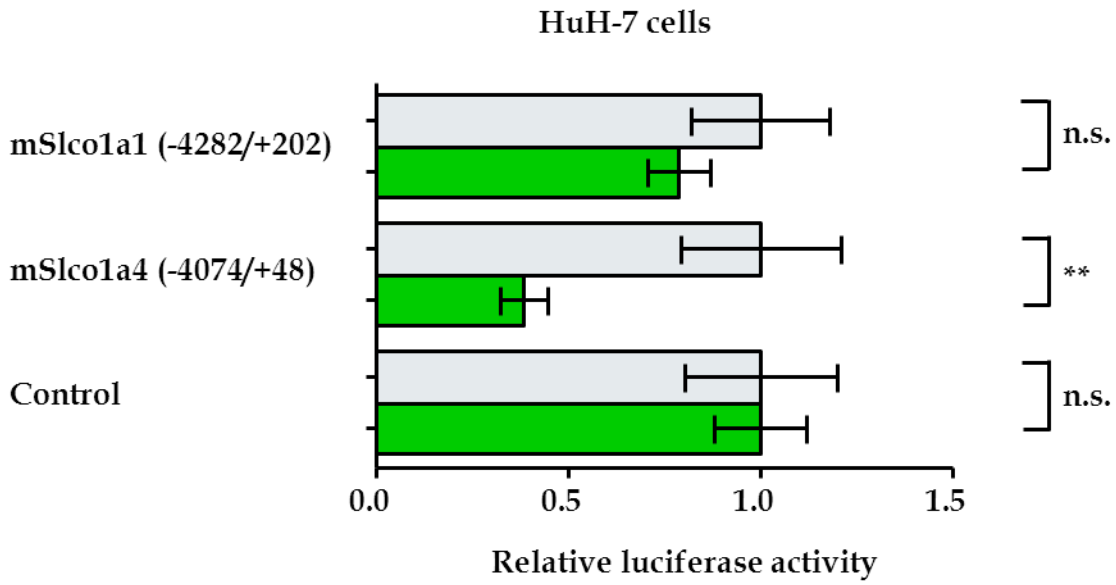
The preferred DNA-binding sequence of FXR is an 'inverted-repeat 1' (IR-1), to which FXR binds as a heterodimer together with RXRα. The ability of this heterodimer to activate the m*Slco1a1* and m*Slco1a4* promoter was examined with co-transfections of TIB-75, HuH-7 and HepG2 cells. Final concentrations of 50 µM chenodeoxycholic acid (CDCA) and 1 µM 9-cis-RA were used to activate FXR and RXRα, respectively. In TIB-75 and HuH-7 cells, the m*Slco1a1* promoter was not activated, whereas the m*Slco1a4* promoter activity was decreased significantly (Fig.

28 A and B). A trend to activation was found in HepG2 cells that, however, was not significant (Fig. 28 C).

A



B



C

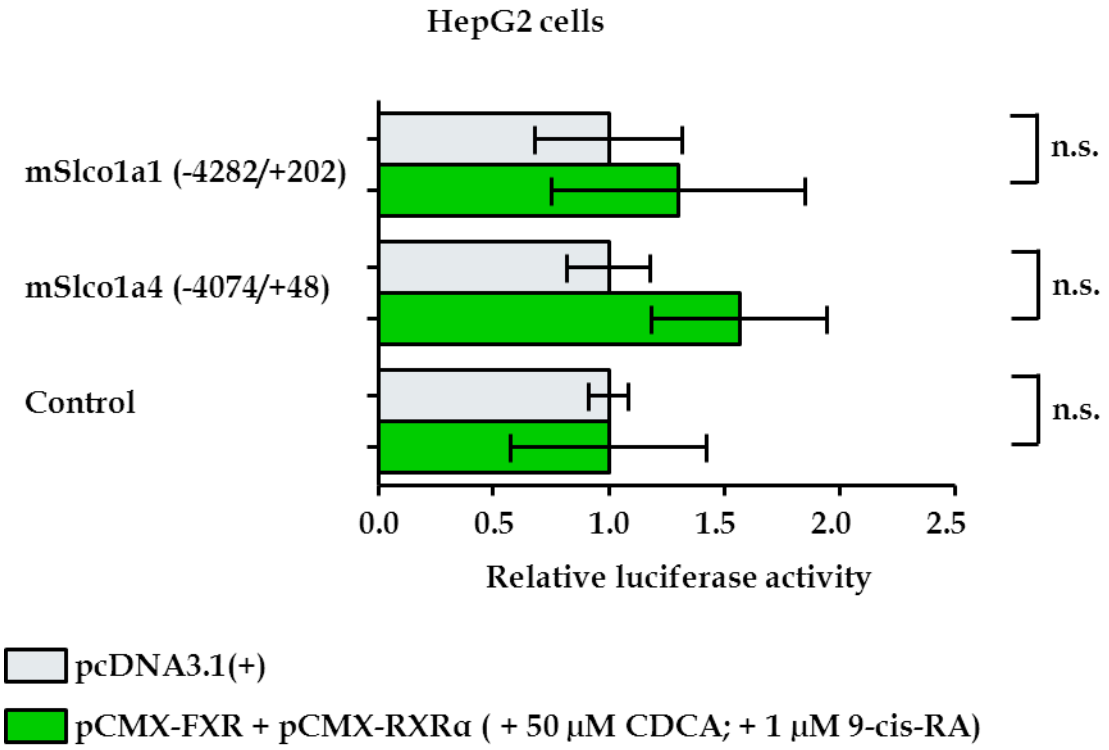


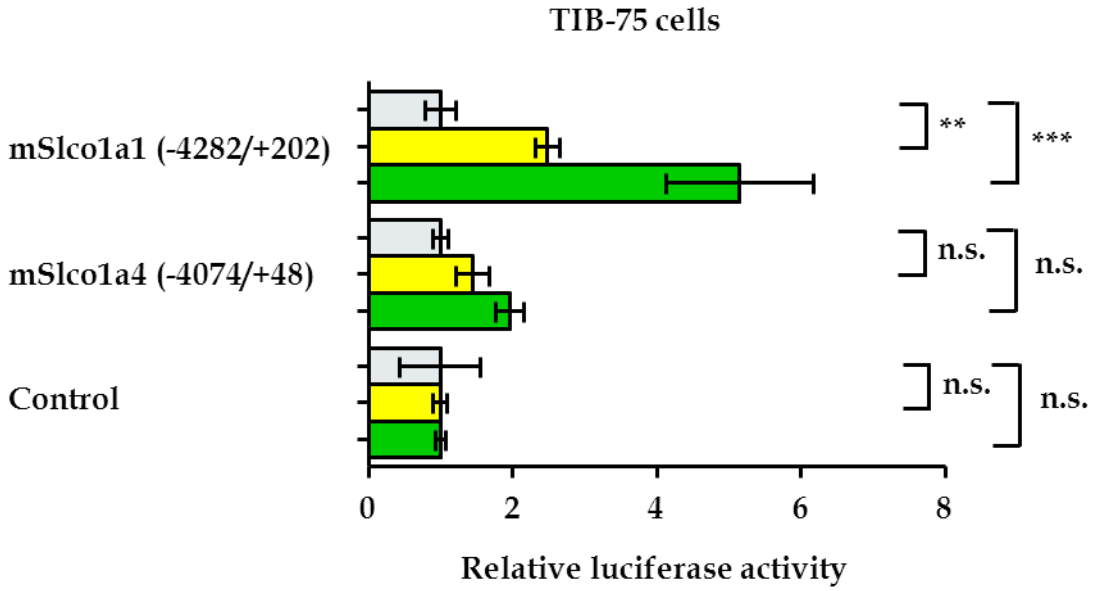
Fig. 28 A - C: Transactivation of the mouse *Slco1a1* and *Slco1a4* promoters by FXR. Final concentrations of 50 µM chenodeoxycholic acid (CDCA) and 1 µM 9-cis-RA were used to activate FXR and RXRα, respectively. **, $p < 0.01$; n.s., not significant.

7.2.7 Transactivation of the mouse *Slco1a1* and *Slco1a4* promoters by mShp

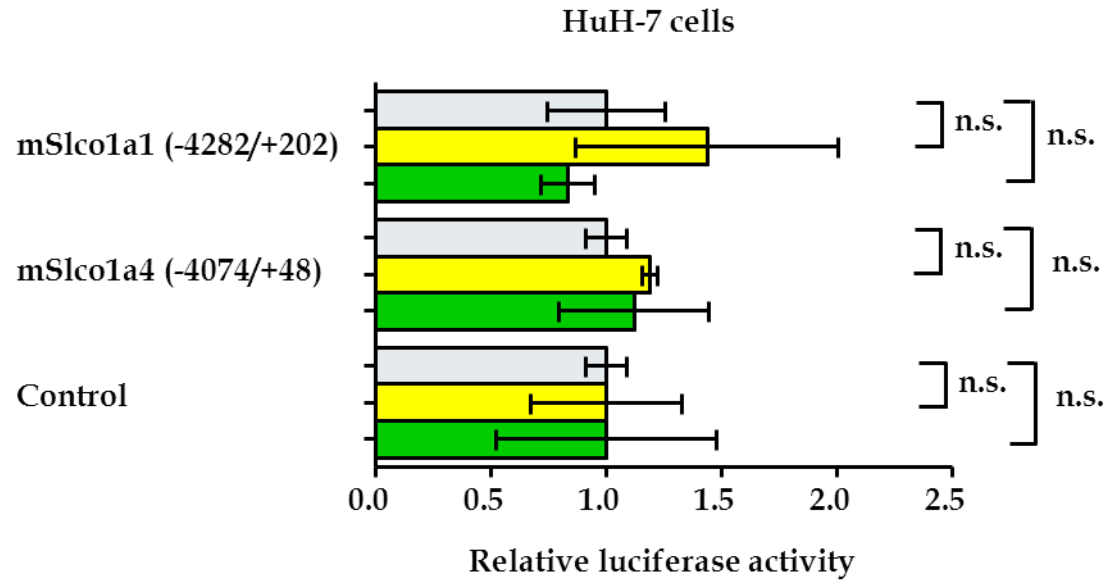
The small heterodimer partner (SHP) contains the dimerization and ligand-binding domain found in transcription factors, but lacks the conserved DNA-binding domain. SHP negatively interacts with other nuclear receptor superfamily members, such as FXR and inhibits transactivation by these receptors. The apoptosis inducer AHPN (6-[3-(1-adamantyl)-4-hydroxyphenyl]-2-naphthalene carboxylic acid) is a known activator of SHP gene expression. The promoter activities in the presence of mouse Shp (mShp) and 5 µM AHPN were studied in TIB-75, HuH-7 and HepG2 cells. The activity of m*Slco1a1* in TIB-75 cells was significantly increased in the presence of mShp alone or with its ligand AHPN (Fig. 29 A), whereas no activation was observed in HuH-7 and HepG2 cells (Fig. 29 B and C). The m*Slco1a4* promoter

did not respond significantly in all three cell lines to either mShp, or mShp and AHPN (Fig. 29 A – C).

A



B



C

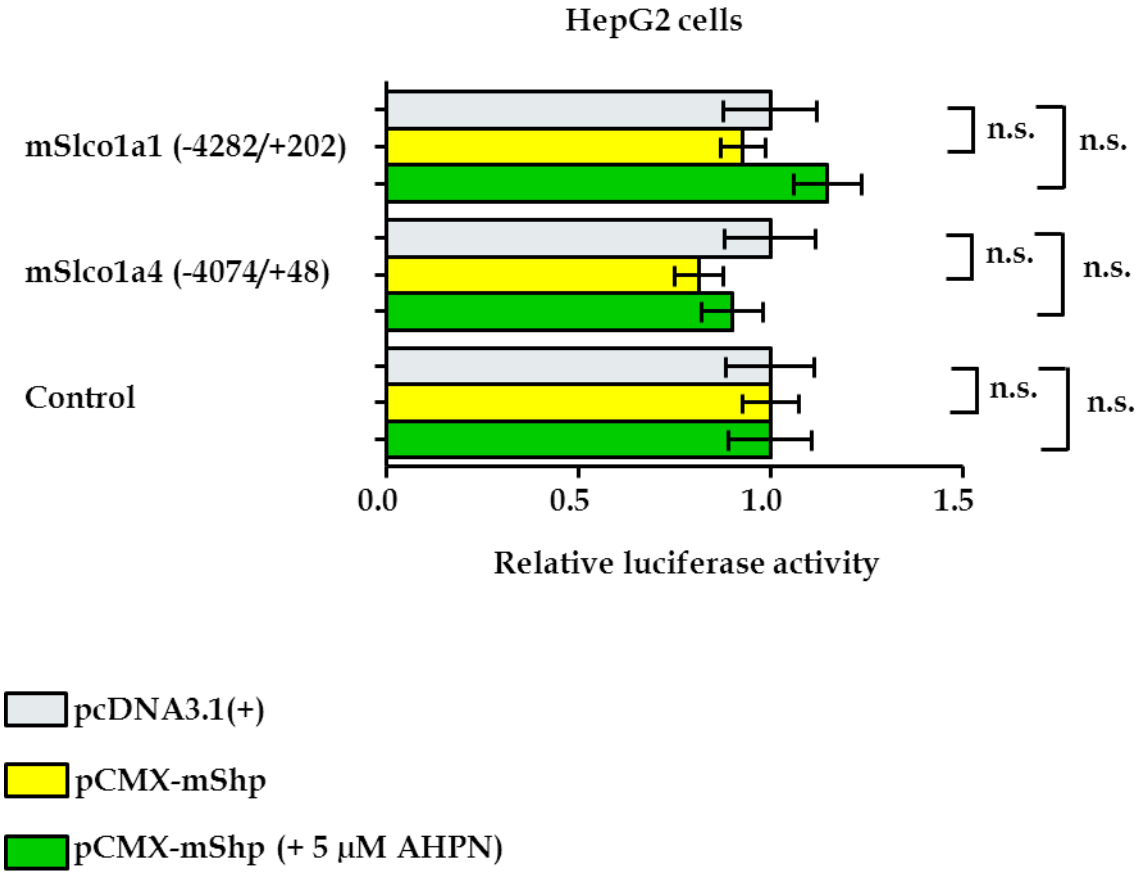


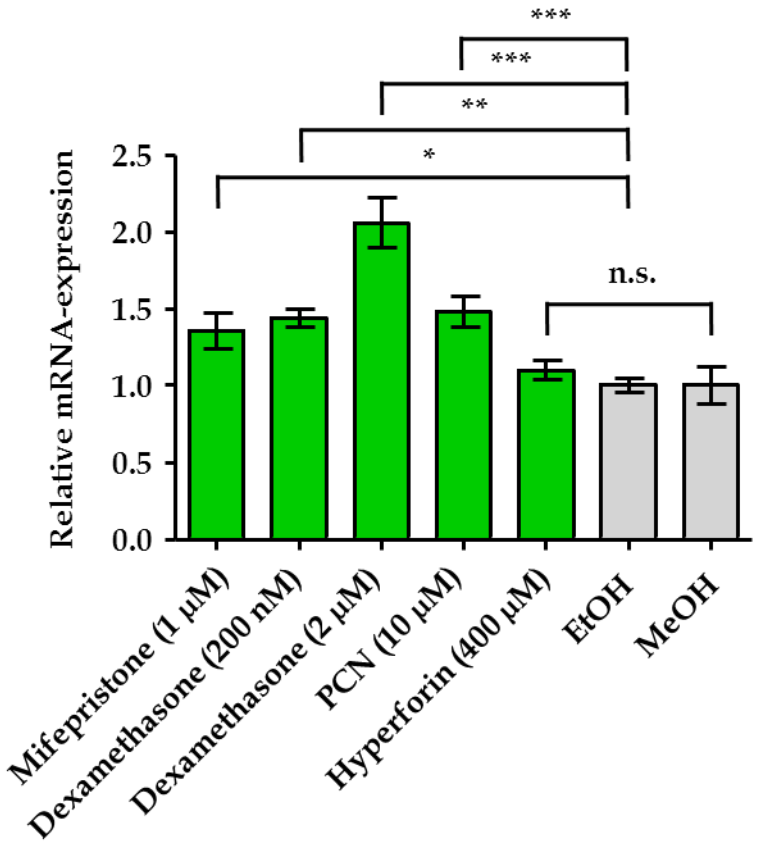
Fig. 29 A - C: m*Slco1a1* and m*Slco1a4* promoter activities in the presence of mShp with or without 5 µM of its ligand AHPN in TIB-75, HuH-7 and HepG2 cells. ***, $p < 0.001$; **, $p < 0.01$; n.s., not significant.

7.2.8 mOatp1a1 and mOatp1a4 mRNA quantification with real-time PCR of mouse liver specimens treated with mPxr and mGr agonists and antagonists

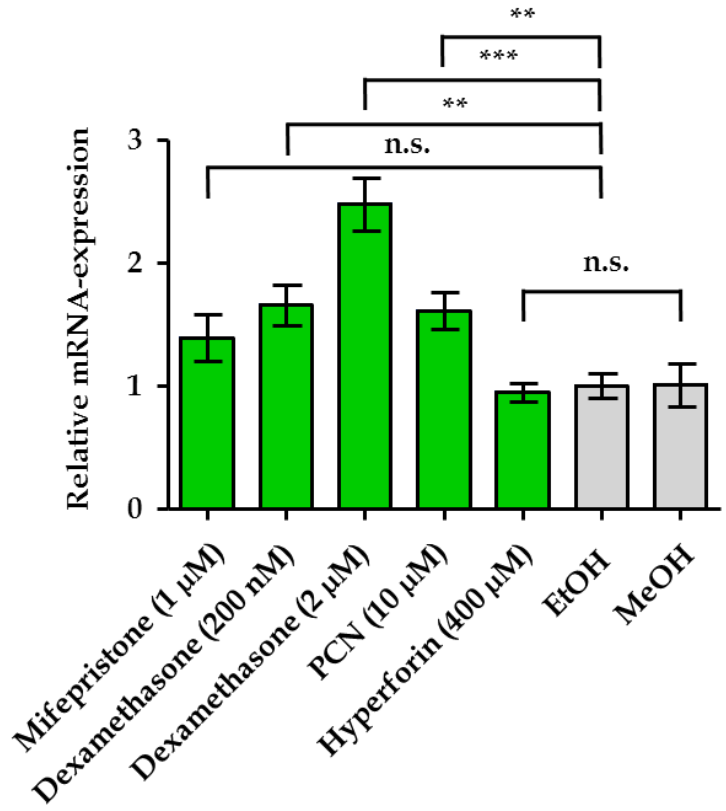
Immortalized cell lines may lose or gain factors or enzymes due to their artificial modifications. Taking this fact into account *ex vivo* samples of mouse livers were taken and treated with ligands of transcription factors. Liver specimens of C57BL/6 and 129S6 mice were incubated in cell culture medium during 4 hours in a CO_2/RH incubator at 37 °C. The incubation medium contained 1 µM mifepristone to inactivate or 200 nM dexamethasone to activate mGr. 2 µM dexamethasone, 10 µM PCN or 400 µM hyperforin were used to activate mPxr. The concentration of

mOatp1a1 mRNA was significantly induced in liver specimens of C57BL/6 mice upon treatment with mifepristone, dexamethasone or PCN, but not upon addition of hyperforin (Fig. 30 A). Dexamethasone and PCN induced the expression of mOatp1a4 mRNA. Both mifepristone and hyperforin did not affect the transcriptional regulation (Fi.30 B). In 129S6 mice, mOatp1a1 mRNA showed increased expression after treatment with dexamethasone and PCN, whereas mifepristone or hyperforin did not change the expression (Fig.30 C). The relative concentration of mOatp1a4 mRNA exhibited no significant changes after the treatments of 129S6 mice liver samples (Fig. 30 D).

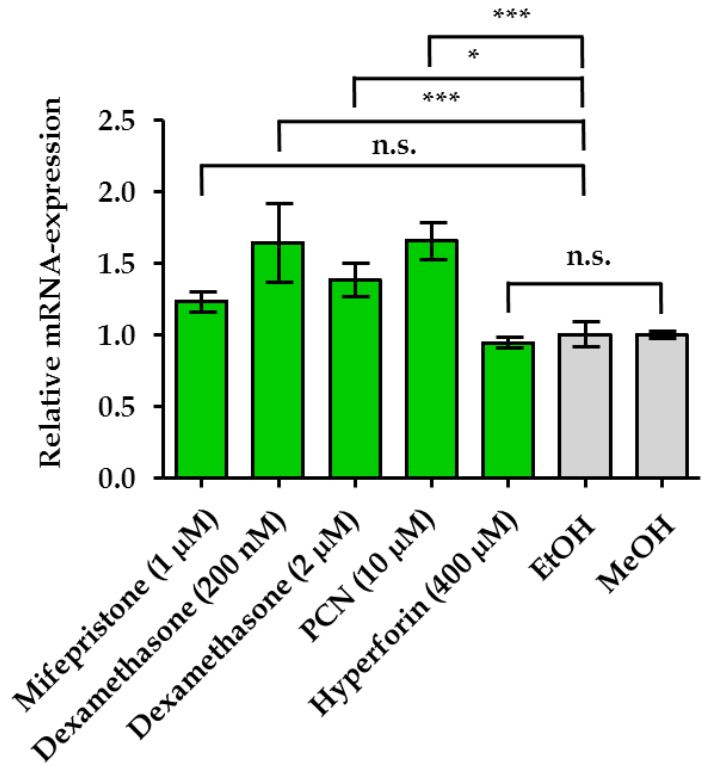
A mOatp1a1mRNA in liver specimens of C57BL/6 mice



B mOatp1a4mRNA in liver specimens of C57BL/6 mice



C mOatp1a1 mRNA in liver specimens of 129S6 mice



D mOatp1a4 mRNA in liver specimens of 129S6 mice

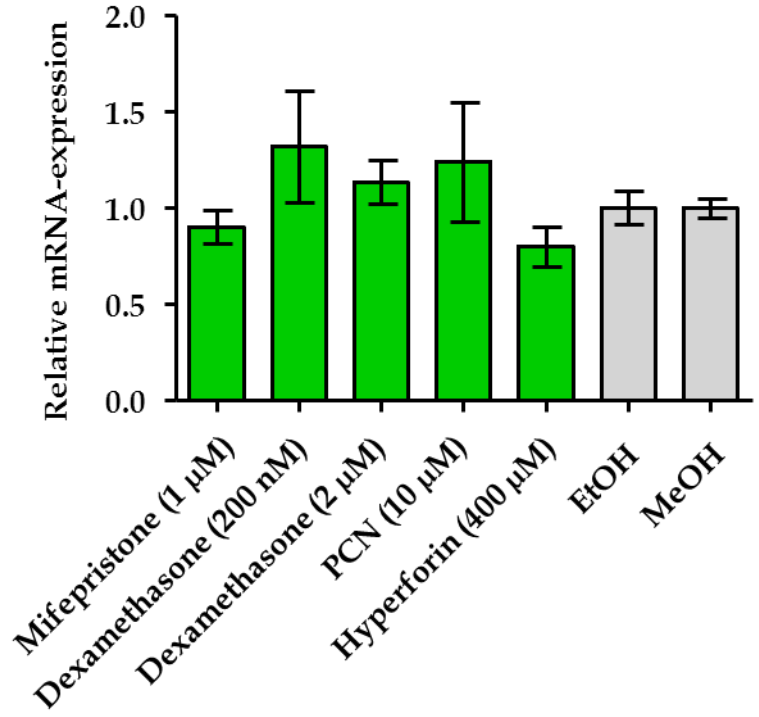


Fig. 30 A-D: Relative mRNA expression of the m*Slco1a1* and m*Slco1a4* genes in liver specimens of C57BL/6 and 129S6 mice. Liver specimens of C57BL/6 and 129S6 mice were incubated in cell culture medium for 4 hours in a CO_2/RH incubator at 37 °C. The incubation medium contained 1 µM mifepristone to inactivate or 200 nM dexamethasone to activate the mGr. To activate mPxr, 2 µM dexamethasone, 10 µM PCN or 400 µM hyperforin were used. ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.1$; n.s., not significant.

8 Discussion

8.1 The peptide transporter 1 (PEPT1/Pept1)

The role of the PEPT1 transporter for the uptake of di- and tripeptides and peptidomimetic drugs from the intestinal lumen underscores the importance of understanding the mechanisms that govern the transcriptional regulation of the *PEPT1* gene. Currently it is known that the transcription of the *PEPT1* gene is regulated by the protein composition of the nutrition (Erickson et al., 1995, Shiraga et al., 1999), starvation (Habold et al., 2007), hormones like thyroid hormones (Ashida et al., 2002 and 2004) and leptin (Buyse et al., 2001), by diabetes (Gangopadhyay et al., 2002), EGF (Nielsen et al., 2001), Ca^{2+} (Wenzel et al., 2002) and cytokines such as TNFα and IFNγ (Vavricka et al., 2006). Shimakura and co-workers showed that the treatment of Caco-2 cells with the PPARα ligand WY-14643 led to increased PEPT1 mRNA levels and an increased uptake of the PEPT1 substrate glycylsarcosine (Shimakura et al., 2006). Furthermore an increase of Pept1 mRNA during fasting was not observed in PPARα knockout mice compared to wildtype mice. These observations suggest a role of PPARα in the transcriptional regulation of PEPT1/Pept1. Another prominent member of the PPAR subfamily of nuclear receptors is PPARγ. It is yet unknown whether PPARγ regulates the expression of PEPT1 or not. Both PPARα and PPARγ bind as heterodimers together with the nuclear receptor RXRα to palindromic response elements. These response elements are characterized as direct repeats of hexamers with one nucleotide as spacer within a promoter of a target gene (Everett et al., 2000, Rosen and Spiegelman, 2001). Considering that both transcription factors bind to the same consensus DNA region, it is not yet fully understood how PPARα and PPARγ differentiate in terms of their specific actions. The nucleotidal composition and the sequence of the nucleotides which form the PPREs may play a role whether a PPARα:RXRα or PPARγ:RXRα heterodimer binds to this element.

In silico analysis of the human and rodent *PEPT1/Pept1* promoters revealed that the human and rat promoter both have a PPRE at nearly the same position upstream of the transcriptional start site, designated (-1512/-1500) in the human and

(-1487/-1475) in the rat promoter. In addition, the human promoter has a second PPRE at position (-401/-389) which could not be found in the rat promoter. The mouse promoter has four PPREs at different locations compared to the human or rat promoter relative to the transcriptional start site.

Currently it is hypothesized that PPARs are located in the cytoplasm in their inactivated state and are bound to repressor proteins (Scher and Pillinger, 2005). The binding of agonistic PPAR ligands leads to the dissociation of these complexes and the PPARs translocate to the nuclei where the interaction with RXRα occurs. Agonistic PPARγ ligands are thiazolidinediones used in the treatment of diabetes mellitus type 2, fibrates regulating hypercholesterolemia , and the naturally occurring 15-deoxy-Δ12,14-prostaglandin J2 (15d-PGJ2). 15d-PGJ2 is a cyclopentenone prostaglandin that contributes like all prostaglandins (PGs) to inflammation, smooth muscle tone, hemostasis, and gastrointestinal secretion, amongst others. PGs are rapidly enzymatically inactivated and act therefore mostly as autocrine or paracrine signaling molecules. PGs exert their inflammatory and anti-inflammatory functions via extracellular or intracellular receptors (Scher and Pillinger, 2005). PGs may be synthesized by the cell itself or taken up from the extracellular space. The transport mechanisms of PGs into and out of a cell are not known in detail. Narumiya and Fukushima showed that murine leukemia cells transport PGs in a Michaelis-Menten kinetic way which indicates an active transport system for PGs (Narumiya and Fukushima, 1986). While most PGs have specific transmembrane receptors, 15d-PGJ2 seems to interact so far only with intracellular receptors such as PPARs (Scher and Pillinger, 2005). First postulated by Ricote and co-researchers, it is today known that 15d-PGJ2 is a ligand of PPARγ (Ricote et al., 1998). After ligand binding of PPARγ and dislocation from the cytoplasma to the nucleus, the complex forms a heterodimer together with RXRα which is activated by 9-cis-RA (Kliewer et al., 1992).

The PPRE sequence found in transcriptional regulatory regions of genes often differ from its perfect consensus AGGTCAnAGGTCA. Comparing the human PPAR response elements at position (-389/-410) and (-1500/-1512) in the regulatory region of the *PEPT1* gene, nine nucleotides of the first PPRE and eight nucleotides of the second PPRE match the perfect consensus sequence. The four PPRE of the mouse

Pept1 promoter have between three and five nucleotides and the PPRE of the rat Pept1 gene has 4 nucleotides matching AGGTCAnAGGTCA. The first guanine and cytosine in the first and second half site of AGGTCAnAGGTCA are conserved between the human PPRE (-389/-402) and the rat PPRE (-1475/-1487) and led to the highest activations in co-transfection assays. This led to the assumption that both PPRE are essential for the overexpression of PEPT1 and rPept1 mRNA.

The loss of activation found for the *PEPT1* (-65/+57) construct in co-transfection assays is consistent with this assumption. In contrast the r*Pept1*(-1048/+20) construct was significantly activated even though *in silico* analysis by MATInspector and NUBIScan and visual search for potential PPREs in this promoter construct was not successful. All m*Pept1* promoter constructs were not significantly activated. The absence in the m*Pept1* promoter of the nucleotides conserved between humans and rats PPREs may be the reason for the non-response in co-transfections of the mouse promoter constructs. EMSAs showed binding of *in vitro* translated PPARγ and RXRα as heterodimers to the human PPRE at position (-1500/-1512) and to the rat PPRE at position (-1475/-1487). The rat PPRE bound quantitatively more PPARγ:RXRα heterodimers than the human PPRE. Both PPREs were not bound by combinations of pSG5 and either PPARγ or RXRα or by pSG5 alone.

The ASBT PPRE used as positive control has the sequence AGGCCAgAGGTCA and has consequently only one nucleotide that is different from the perfect PPRE sequence. The four selected mouse PPREs did not interact with any *in vitro* translated protein. The EMSA results obtained support the observations made in co-transfection assays that the human and rat *PEPT1* promoter but not the mouse promoter interacts with PPARγ:RXRα heterodimers.

In a further step Caco-2 cells were treated with 15d-PGJ2 and 9-cis-RA to activate PPARγ and RXRα followed by an extraction of these protein complexes from the nuclei. These nuclear protein extracts were used instead of *in vitro* translated proteins in EMSAs to examine the interaction of PPARγ:RXRα heterodimers from living cells with the DNA. The visualization of the protein complexes bound to the PPREs displayed so-called smearing bands indicating that not a single protein type or protein complex binds to the DNA but several different proteins or protein

complexes. It may be that the PPREs were bound by other proteins or protein complexes different from the PPARγ:RXRα heterodimers. The use of PPARγ or RXRα antibodies did not result in a supershift of bands which means that the antibodies did not interact with the nuclear extracts. Both PPARγ and both RXRα antibodies should interact with their antigens according to the literature (Lii et al., 2012, Yuan et al., 2012, Childs et al., 2011, Adhikary et al., 2011).

A further step to gain insight into a possible binding of PPARγ:RXRα heterodimers to PPREs in the *PEPT1*/r*Pept1* promoters is to mutate nucleotides of the PPREs which are essential for the binding of PPARγ:RXRα heterodimers (Okuno et al., 2001). Point mutations exchanged these nucleotides of the human PPREs (-389/-401) and (-1500/-1512) and the rat PPRE (-1475/-1487), and the mutated PPREs and promoter constructs were tested for their abilities to bind PPARγ:RXRα heterodimers. In co-transfection assays there was no decrease in activation of promoter constructs featuring either one or all mutated human and rat PPREs compared to the promoter constructs with the wild-type PPREs. PPARγ:RXRα heterodimers either still interacted to a sufficient degree with the mutated DR-1 sequence or alternatively bound to a DR-1 like element at a different position not affected by the mutation.

To study a possible interaction between the *PEPT1* promoter and the transcription factors PPARγ and RXRα within living cells, the endogenous relative mRNA levels of the genes of interest were first determined in Caco-2, DLD-1, T84, LS174T and HT-29 cells to select a suitable cell line. Caco-2 cells expressed by far the highest concentrations of PEPT1 mRNA followed by T84 cells. DLD-1, LS174T and HT-29 had very low or even undetectable levels of PEPT1 mRNA. Even though PEPT1 mRNA is found primarily only in the small intestine, the human colon derived cell line Caco-2 expresses the PEPT1 transporter (Brandsch et al., 1994). The PEPT1 mRNA detection in Caco-2 cells is in consistence with the study of Brandsch and co-workers. The differences in the expression of PPARα, PPARγ and RXRα mRNA were not as distinctive as of PEPT1 mRNA in the cell lines. PPARα was included in the studies to examine the possibility of a PPARα interaction with the *PEPT1* promoter as a heterodimer together with RXRα. PPARα:RXRα binds like PPARγ:RXRα heterodimers to DR-1 motifs of the DNA and regulates the

transcription of genes (Keller et al., 1993). The Caco-2 cells were chosen as cell line for further studies because of the high PEPT1 mRNA levels and the similar expression of PPARα, PPARγ and RXRα mRNA expression levels compared to the other cell lines. Additionally, Caco-2 cells were already used in co-transfection assays.

The 48-hour incubation of Caco-2 cells with the agonists of RXRα, 9-cis-RA, the agonist of PPARγ, 15d-PGJ2, and the antagonist of PPARγ, GW-9662, alone showed no alterations in the PEPT1 mRNA levels. The combination of 15d-PGJ2 and GW-9662 did not change the expression, whereas the combination of 15d-PGJ2 and 9-cis-RA led to lower levels of PEPT1 mRNA compared to the cells treated with the solvent of the ligands. Natural agonists for PPARγ are free fatty acids (Dreyer et al., 1993). They are components of bovine serum which is a supplement in cell culture to support the proliferation of tissue cells. To exclude an activation of endogenous PPARγ by these fatty acids, delipidated serum was used in a further experiment. The mRNA levels of PEPT1 showed still the same levels as the result obtained with cells treated with normal cell culture medium. On the one hand, there are two main problems with incubations over a 48-hour time period that can occur caused by the different dynamics of the participating compounds involved in the regulation. First, the ligands used to stimulate the transcription are rapidly deactivated, which is the case for many prostaglandins like 15d-PGJ2. Second, an overexpression of a certain gene by endogenous or exogenous compounds may activate feedback mechanisms down-regulating the 'abnormal' expression over time. On the other hand, the transcriptional machinery needs a certain time period to be activated and to synthesize the mRNA.

These considerations resulted in an experiment in which a reduction of the incubation period to 24 hours was tested. This experiment showed the importance of the incubation period for the mRNA levels detected which were rather different from the 48 hours treatment. Caco-2 cells cultured in normal tissue medium and treated with 15d-PGJ2 alone or in combination with the antagonist GW-9662 had the tendency to higher PEPT1 mRNA levels rather than the control cells. 9-cis-RA alone or in combination with 15d-PGJ2 had lower levels, and GW-9662 alone resulted in PEPT1 mRNA levels that were not significantly different from the control. Cells in delipidated cell culture medium and treated with the antagonist GW-9662 alone

developed significantly higher PEPT1 mRNA levels. All other treatments resulted in lower or unchanged PEPT1 mRNA levels. Due to these inconsistent results analysis of the exact role of PPARγ and RXRα in the transcriptional regulation of PEPT1 in Caco-2 cells will require further investigation.

Even though Caco-2 cells express PPARγ and RXRα at certain levels shown by mRNA quantification, expression plasmids that contained the coding sequences of both transcription factors were transfected into Caco-2 cells to enhance intracellular concentrations of PPARγ and RXRα proteins. Twenty four hours after the transfection, the agonistic ligands of PPARγ and RXRα, 15d-PGJ2 and 9-cis-RA, respectively, were added to the medium and the PEPT1 mRNA levels were measured after another twenty four hours. The addition of 15d-PGJ2 led to a significant increase in PEPT1 mRNA compared to the cells treated with the solvent of 15d-PGJ2. The combination of 15d-PGJ2 and 9-cis-RA augmented the concentration of PEPT1 mRNA compared to the cells treated with 15d-PGJ2 only. The addition of 9-cis-RA alone had no influence on the PEPT1 mRNA levels.

According to Jung and co-workers, PPARα regulates together with its heterodimeric partner RXRα the expression of ASBT mRNA in Caco-2 cells (Jung et al., 2002). This was confirmed in co-transfection assays of the ASBT promoter and PPARα and RXRα expression plasmids, in EMSAs with *in vitro* translated PPARα and RXRα and by induction of ASBT mRNA expression via addition of the PPARα agonist ciprofibrate to SK-ChA cholangiocytes. Due to the fact that both PPARα:RXRα and PPARγ:RXRα heterodimers bind DR-1 like motifs in promoter sequences, the ASBT mRNA levels of the treated cells were used as positive control. The PEPT1 mRNA levels of the cells treated with 15d-PGJ2 and 9-cis-RA increased ~2 fold, whereas the addition of the ligands alone showed an increase in PEPT1 mRNA of maximum ~1.3 fold. Despite several lines of evidence that indicate an induction of PEPT1 mRNA transcription in Caco-2 cells co-transfected with PPARγ and RXRα expression plasmids, some aspects regarding the interaction of PPARγ:RXRα heterodimers and the *PEPT1* promoter remain unclear.

Another assay to analyze the influence of transcription factors on gene expression would be to knockdown certain transcription factors and as a consequence reduce the transcription of genes regulated by the affected transcription

factors. For these assays Caco-2 cells were used because they express RXRα, PPARα and PPARγ proteins at detectable levels. The knockdown of all three transcription factors alone or in combination was verified by real-time PCR. To test the functioning of the method, first mRNA levels of ASBT were detected in Caco-2 cells treated with RXRα and PPARα siRNA. The mRNA levels of ASBT in Caco-2 cells treated with RXRα and PPARα siRNA were significantly lowered, which confirms the hypothesis of Jung and co-researchers (Jung et al., 2002). However the knockdown of RXRα, PPARα, PPARγ and its two heterodimeric variations did not alter the expression of PEPT1 mRNA.

HNF4α is a liver-enriched nuclear transcription factor and interacts with DR-1 motifs, but, unlike the PPARs, it forms a homodimer which binds to DNA (Jiang et al., 1995). A possible interplay of HNF4α and RXRα, PPARα and PPARγ was examined with siRNA targeting these transcription factors in Caco-2 cells. This knockdown experiment showed a slight, but not significant, decrease of PEPT1 mRNA in Caco-2 cells treated with 80 nM concentrations of HNF4α siRNA and 80 nM and 40 nM concentrations of PPARα and RXRα siRNA, respectively. Lower concentrations of HNF4α siRNA or combinations with PPARγ siRNA did not have an influence on the expression of PEPT1 mRNA. The unchanged PEPT1 mRNA in Caco-2 cells treated with the siRNA targeting the transcription factors as compared to the concentration of PEPT1 mRNA in cells treated with non-targeting control siRNA led to the assumption that neither PPARα and PPARγ nor RXRα and HNF4α has an important role in the transcriptional regulation of PEPT1.

A possible interaction between rat Rxrα, Pparα and Pparγ and the rat *Pept1* promoter was analyzed by treating *ex vivo* ileal specimens of rats with corresponding agonists and antagonists. *Ex vivo* treatment of intestinal rat specimens was previously successfully established in our laboratory (Eloranta et al., 2009). Duodenal rat specimens were treated during eight hours with vitamin D_3 which resulted in an increased expression of proton coupled folate transporter mRNA. To analyze the rat Pept1 mRNA expression, the specimens were incubated in medium containing agonists and/or antagonists of RXRα, PPARα and PPARγ. PPARα agonists WY-14643 (Reddy et al., 1978) and ciprofibrate (Auwerx, 1992) and the antagonist MK-886 (Kehrer et al., 2001) were examined. Troglitazone (Lambe and Tugwood, 1996), 15d-

PGJ2 and GW-1929 (Brown et al., 1999) were used to activate PPARγ. GW-9662 (Huang et al., 1999) was applied to inhibit the activation of PPARγ. All agonists and antagonists of PPARα and PPARγ were added alone or in combination with the agonist of RXRα to the medium.

Surprisingly, all treatments resulted in an increased expression of rat Pept1. Addition of the antagonists of PPARα and PPARγ led to similar or even higher Pept1 mRNA expression than addition of the agonists. The highest activation was observed when 9-cis-RA was exclusively added. As positive control, the collected mRNA samples were analyzed for their Asbt mRNA expression. The pattern of mRNA concentration resembled the concentration of Pept1. These inconsistent results may be the consequence of different stabilities of mRNAs of different genes (Kren and Steer, 1996). The rate of poly(A) tail removals or the binding of proteins to the mRNAs may result in increased or decreased transcript stability. In addition the cells are exposed from the moment of taking the sample to extreme stress condition even when they are incubated in tissue cell incubators with adjusted temperatures and CO_2/RH conditions for the cells. These conditions may also contribute to an altered mRNA stability.

8.2 The organic anion transporting polypeptides (Oatps)

The organic anion transporting polypeptides (SLCO gene family) are characterized by a broad substrate specificity and expression in epithelial cells of various tissues. The factors that influence their expression level are of great interest due to their potential effect on the pharmacokinetics of the substrates transported (for references see introduction)

In silico analysis revealed that the mouse *Slco1a1* promoter covered two DR-3 response element and one DR-4, DR-6 and IR-3 response element, whereas the mouse *Slco1a4* promoter covered one DR-1, DR-4, DR-9, DR-10, ER-2 and IR-3 response element . The glucocorticoid receptor homodimer complex interacts with different variations of palindromic consensus sequences with the half site TGTTCT (Aumais et al., 1996). These include direct repeats (DR), everted repeats (ER) and inverted repeats (IR) of the half site with different numbers of nucleotides as spacer

in between. It is known that the mouse *Slco1a1* promoter interacts with Hnf1α, Hnf4α and Fxr, but whether the glucocorticoid receptor binds to any of the mentioned response elements is not known. First results of co-transfection assays in the mouse liver-derived cell line TIB-75 showed a significant activation of the m*Slco1a1* (-1991/+202) construct covering the DR-3, DR-4 and DR-6 response element, whereas the m*Slco1a1* (-553/+202) construct covering the DR-3 and DR-6 response element did not respond to the administration of the human GR and its ligand dexamethasone. This would implicate an activation of the m*Slco1a1* (-1991/+202) construct by the GR. However, the m*Slco1a1* (-439/+202) construct covering no known GR response element was activated to the same level by GR as m*Slco1a1* (-1991/+202). As positive control the activation of a promoter construct of NTCP by GR was confirmed (Eloranta et al., 2006). The activation of the m*Slco1a4* constructs covering the DR-1, DR-4, ER-2 and IR-3 response elements by the GR were measured as well in TIB-75 cells. Surprisingly the shortest promoter construct covering no known GR response element was activated with highest significance and the promoter constructs covering at least one GR response element were not responsive. However this result may be questionable due to the positive control promoter construct which showed not the normal activation by GR.

Repetition of both assays in human liver-derived cells, HuH-7, showed a non-significant activation of the m*Slco1a1* (-1991/+202) construct and a significant activation of the positive control, whereas all other mSlco1a1 promoter constructs were not activated by GR. The m*Slco1a4* promoter constructs did not respond to the treatment with GR and its ligand, but the positive control was activated as previously shown. So far, the replacement of the mouse liver cell line with a human liver cell line did not result in an activation of the m*Slco1a1* or m*Slco1a4* promoter constructs by GR. To avoid a possible incompatibility of the GR with the mouse specific cell line TIB-75 or the mouse promoter constructs tested, the mouse specific glucocorticoid receptor was cloned and tested in co-transfection assays. In these assays the longest promoter constructs of m*Slco1a1* and m*Slco1a4* were analyzed instead of the shorter constructs used previously. The longest m*Slco1a1* (-4282/+202) promoter construct covered in addition to the m*Slco1a1*

(-1991/+202) construct a DR-3 and IR-3 response element, whereas the longest m*Slco1a4* (-4074/+48) construct covered additionally to the m*Slco1a4* (-3832/+48) construct a DR-9 and DR-10 response element. In TIB-75 cells, the m*Slco1a1* (-4282/+202) construct was significantly activated, whereas the m*Slco1a4* (-4074/+48) construct did not respond to the addition of the mGr and its ligand dexamethasone. This is consistent with the results obtained by co-transfection of the shorter constructs and the GR in TIB-75 cells. In HuH-7 cells both promoter constructs were significantly activated, which is in contrast to the results with the shorter constructs in the same cell line.

Another prominent transcription factor possibly regulating both promoters is the PXR due to its binding to DR-3, DR-4 and ER-6 response elements. Both promoter constructs were tested in HuH-7 and TIB-75 cells with increasing PCN concentrations and the mouse isoforms of PXR, mPxr, and RXRα, mRxrα. The m*Slco1a1*(-4282/+202) construct was significantly activated, whereas the m*Slco1a4* (-4074/+48) construct had a tendency to higher activity when treated with high concentrations of PCN in TIB-75 cells. In HuH-7 cells, only the m*Slco1a1* (-4282/+202) promoter was activated significantly by the highest and lowest PCN concentration. Co-transfections in a further human liver-derived cell line, HepG2, showed that both the longest m*Slco1a1* and m*Slco1a4* promoter constructs were activated by mGr and mPxr:mRxrα but not in a significant manner. An involvement of human FXR together with its heterodimeric partner RXRα or the mShp protein in the presence of its ligands CDCA, 9-cis-RA or AHPN in the regulation of both promoters could not be shown in co-transfection assay in TIB-75, HuH-7 and HepG2 cells.

Liver samples of 2 strains of mice, C57BL/6 and 129S6, with agonists and antagonists of Gr and Pxr were treated *ex vivo* during four hours. In C57BL/6 mice, both the agonist and the antagonist of Gr induced the mRNA expression of mSlco1a1. The activation of Pxr by 2 µM dexamethasone and 10 µM PCN led to higher concentrations of mSlco1a1 mRNA, whereas the addition of the Pxr agonist hyperforin did not have any effect on the mRNA expression of the m*Slco1a1* gene. The same pattern of increased mRNA concentrations was observed for the m*Slco1a4* gene except for mifepristone which did not affect the expression of the m*Slco1a4* gene significantly. The mSlco1a1 mRNA expression was induced in 129S6 mice liver

specimens by low and high doses of dexamethasone and PCN, but not by mifepristone or hyperforin. The mRNA of the m*Slco1a4* gene was not significantly increased in the same samples.

Some results from co-transfection assays or the *ex vivo* treatment of mouse liver specimens would indicate an interaction between the mentioned transcription factors and the m*Slco1a1* or m*Slco1a4* promoters. Nevertheless, a consistent activation of m*Slco1a1* or m*Slco1a4* promoters by PXR/RXRα, mPxr:mRxrα, GR, mGr, FXR or mShp was not observed in co-transfection assays or treatment of mouse and human liver-derived cell lines with the respective ligands of the transcription factors.

9 Future directions

The present thesis gives more details into the mechanisms that control the transcriptional regulation of the human and rodent *PEPT1/Pept1* gene promoters and the mouse *Slco1a1* and *Slco1a4* gene promoters.

The described experiments have shown for the first time that PPARγ influences together with RXRα the activities of the human and rat *PEPT1/Pept1* promoters. The results obtained point to an involvement of the PPARγ:RXRα heterodimer in the transcriptional regulation of the human and rat *PEPT1/Pept1* genes. Although PPARγ:RXRα significantly changed the human and rat *PEPT1/Pept1* promoter activities, it remains to be elucidated whether other transcription factors or signaling pathways contribute to this regulation cascade. Further assays are needed to pursue the findings of this study. One point which should be considered in future experiments is the effect of PPARγ agonists on the PEPT1 protein expression in Caco-2 cells. Such experiments would give more details about the functional transport of PEPT1 substrates. Although the changes of PEPT1 promoter activity were significant, it is questionable whether these regulations are sufficient to fully explain the activity changes of the PEPT1 protein in the apical membrane of enterocytes. It should be taken into account that regulations at many steps from the transcription to the integration of the PEPT1 proteins into the phospholipid bilayers are possible.

The examination of the mouse *Slco1a1* and *Slco1a4* promoters lead to the conclusion that the activation of the glucocorticoid receptor by dexamethasone has an effect on the transcriptional regulation of both promoters. This was shown in co-transfection assays measuring the *Slco1a1* and *Slco1a4* promoter activities by luminescence and by treating explants of mouse liver with dexamethasone. These results show for the first time that the transcriptional regulation proceeds via the glucocorticoid receptor mediated pathway. To acquire more knowledge about the involvement of the glucocorticoid receptor in the signaling cascade that activates or represses the *Slco1a1* or *Slco1a4* promoter more detailed molecular analyses are needed.

10 Contributions to original articles

- **Eloranta JJ, Hiller C, Jüttner M, Kullak-Ublick GA (2012) The SLCO1A2 gene, encoding human organic anion-transporting polypeptide 1A2, is transactivated by the vitamin D receptor. Mol Pharmacol 82:37-46**

The first Western blot showing an induction of OATP1A2 expression by treatment of Caco-2 cells with vitamin D_3 was initially done by M Jüttner (Figure 1 D). The transfection assays with siRNA specifically targeting VDR in Caco-2 cells shown in Figure 2 A and B were established, performed and analyzed by M Jüttner. Preliminary experiments showing with ChIP assays the interaction of VDR with the vitamin D_3-responsive region of the *SLCO1A2* promoter within Caco-2 cells were done by M Jüttner (Figure 5).
Several transport experiments with OATP1A2 substrates like deltorphin II, enkephalin and estrone-3-sulfate were performed to evaluate a potential change of OATP1A2 transport activity upon treatment of Caco-2 cells with vitamin D_3. There was a tendency to an increased transport activity of OATP1A2 substrates into Caco-2 cells treated with vitamin D_3. The data were finally not included in the manuscript.

- **Ma L, Jüttner M, Kullak-Ublick GA, Eloranta JJ (2012). Regulation of the gene encoding the intestinal bile acid transporter ASBT by the caudal-type homeobox proteins CDX1 and CDX2. Am J Physiol Gastrointest Liver Physiol 302:G123-33**

The co-transfections assays in Caco-2 and IEC-4.1 cells comparing the human and rodent *ASBT*/*Asbt* promoter activation by CDX1 and CDX2 were performed and analyzed by M Jüttner (Figure 2 F and G).

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12 Curriculum vitae

JÜTTNER
MORITZ PHILIPP
14 April 1981
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Education and work experience

- 2001-2005 study of pharmacy at the University of Basel and diploma examination

- 2005 diploma thesis at the Institute of Molecular Pharmacy, Department of Pharmaceutical Science (Prof. Beat Ernst) with the topic "Directed evolution of bifunctional sialyltransferase" (dipl.pharm.)

- 2006 qualification as a federal pharmacist (eidg.dipl.pharm.) at the University of Basel

Current position (since 1 April 2008)

- Doctoral thesis work (PhD) at the University Hospital Zurich, Division of Clinical Pharmacology and Toxicology (Prof. Gerd Kullak-Ublick) with the topic "Transcriptional regulation of intestinally and hepatically expressed membrane transporter genes".

Conference presentations

• May 2008: Poster presentation at the Transporters Meeting in Murten (CH): Regulation of the gene encoding the intestinal bile acid transporter ASBT/Asbt by the transcription factor CDX1 (Li Ma, Moritz Jüttner, Jyrki J. Eloranta, and Gerd A. Kullak-Ublick)

• December 2008: Oral presentation at the Young Investigators' meeting for Research in GI and Liver Diseases in Konolfingen (CH): Comparative Analysis of the Human, Mouse, and Rat *ASBT/Asbt* Promoter Regulation

• November 2008: Oral presentation at the ZIHP collaborative project meeting: Transcriptional regulation and functional characterization of intestinal transport of drugs, peptides, and bile acids in mice, rats, and humans

• April 2009: Poster presentation at the 8th Day of Clinical Research of the University Hospital Zurich: Comparative analysis of the human, mouse, and rat *ASBT/Asbt* promoter regulation (Moritz Jüttner, Jyrki J. Eloranta, and Gerd A. Kullak-Ublick)

• July 2009: Poster presentation at the Pharmacology Poster Day: Regulation of the human and rodent *ASBT/Asbt* and *PEPT1/Pept1* promoters by PPARα and PPARγ (Moritz Jüttner, Jyrki J. Eloranta, and Gerd A. Kullak-Ublick)

• August 2009: Poster presentation at the 5th ZIHP Symposium: Comparative analysis of the human, mouse, and rat *ASBT/Asbt* and *PEPT1/Pept1* promoter regulation (Moritz Jüttner, Jyrki J. Eloranta, and Gerd A. Kullak-Ublick)

• April 2010: Poster presentation at the 9th Day of Clinical Research of the University Hospital Zurich: Comparative analysis of the human, mouse, and

rat *ASBT*/*Asbt* and *PEPT1*/*Pept1* promoter regulation (Moritz Jüttner, Jyrki J. Eloranta, and Gerd A. Kullak-Ublick)

• June 2010: Poster presentation at the Pharma Poster-Day: Comparative analysis of the human, mouse, and rat *ASBT*/*Asbt* and *PEPT1*/*Pept1* promoter regulation (Moritz Jüttner, Jyrki J. Eloranta, and Gerd A. Kullak-Ublick)

• August 2010: Poster presentation at the 6th Symposium of the ZIHP: Comparative analysis of the human, mouse, and rat *ASBT*/*Asbt* and *PEPT1*/*Pept1* promoter regulation (Moritz Jüttner, Jyrki J. Eloranta, and Gerd A. Kullak-Ublick)

• May 2012: Poster presentation at the Digestive Disease Week (DDW) in San Diego, USA: The gene encoding the human organic anion transporting polypeptide OATP1A2 (*SLCO1A2* gene) is transactivated by the vitamin D receptor (VDR) (Moritz Jüttner, Jyrki J. Eloranta, Christian Hiller and Gerd A. Kullak-Ublick)

• January 2013: Oral presentation at the 12th Hepatobiliary and Gastrointestinal Research Retreat in Vulpera, Switzerland: The gene encoding the human organic anion transporting polypeptide 1A2 (OATP1A2, gene symbol *SLCO1A2*) is transactivated by the vitamin D receptor (VDR) (Moritz Jüttner, Jyrki J. Eloranta, Christian Hiller and Gerd A. Kullak-Ublick)

Courses attended

• October 2008: Education to carry out animal experimentations (category FELASA B) at the Institute of Laboratory Animal Science of the University of Zurich

• October 2008: Attendance at the IBD-net Clinical Investigators' Day: How to design a clinical trial

• December 2008: Attendance at the 9th Annual Meeting of the centre for Xenobiotic and Environmental Risk Research (XERR)

• November 2008/March 2009/November 2009/April 2010: Tutoring at the Institute of Physiology Zurich

• September 2009: Attendance of the course: LTK Module 14E: Current topics in Laboratory Animal Science

• February 2010: Postgraduate Course of the PhD Program in Integrative Molecular Medicine (imMed) 'Electrophysiology'

• March 2010: Participation in the advanced education course 'Mouse physiology and pathophysiology'

• October 2012: Participation in the education course 'Scientific writing in the Sciences and Medicine'

• October 2012: Participation in the education course 'Lösungsorientiertes Stressmanagement'

• November 2012: Participation in the education course 'Wissenschaft öffentlich kommunizieren'

13 Appendix

Sequences of oligonucleotides used for cloning, site-directed mutagenesis and EMSAs

The introduced restriction sites are underlined. The corresponding restriction enzyme used is listed for each oligonucleotide. Only the top strands (+) are shown for oligonucleotides used in cloning and EMSAs. Both strands (+/-) are shown of the oligonucleotides used in site-directed mutagenesis. Point mutations compared to the wildtype sequence are red labeled.

- **Cloning oligonucleotides**

PEPT1 promoter construct

RP:
ACG CGT GGC GGC GGC TCC CAG GGC (MluI)

FP (-2010):
GAG CTC GTG CCT CAG TAA ACA TAT GTG TGC ACG TG (SacI)

m*Pept1* promoter construct

RP:
CTC GAG CGA CTG GCA AGC AGG AGT TCA G (XhoI)

FP (-1095):
ACG CGT GAA TAT GGC TGA GCC TGG AGA ACA C (MluI)

FP (-2150):

ACG CGT CAA ATT CCC CTC CTC AAC CTC CC (MluI)

r*Pept1* promoter constructs

RP:

CTC GAG ACT GGC AAG CAG GAG TTC AGC C (XhoI)

FP (-1048):

ACG CGT TGC TAA CTG AAA GAA GCC AGC CAC AG (MluI)

FP (-1943):

ACG CGT GAT TTG TTA GAG AAA GAA GCC CAG TGG (MluI)

m*Slco1a1* promoter constructs

RP:

CTC GAG GAT AGA GTG CCT TAG TCA GAG TTT C (XhoI)

FP (-439):

GCT AGC GTC TCT TCA TTC AGT TTC TTG GTG GT (NheI)

FP (-553):

GCT AGC TCT GTC CAT AAG TAT GTG TGC AGG TG (NheI)

FP (-1991):

GCT AGC AGA GCA AGT TTA GAT ATC AAA TCA GCA AC (NheI)

FP (-3926):

GCT AGC AAT CCC AGC ACT TTG GAG GCA G (NheI)

FP (-4282):

GCT AGC GCA CTG GAA ATG TTA ATG AAG GAA ATA CC (NheI)

m*Slco1a4* promoter constructs

RP:

CTC GAG CAA GTG AGA AGT CCA CAC ATG AAG (XhoI)

FP (-1013):

GCT AGC GAA CTG AGT GGT CTG TCT AAA CAC AC (NheI)

FP (-1163):

GCT AGC TCT CTT CCT GAT ACA GCC TTT AAC G (NheI)

FP (-1227):

GCT AGC CCA TCC TAG CTT CCA ATG GTG G (NheI)

FP (-1672):

GCT AGC GTA GGG CTG AAT TCA TGA ATC TCA CTC (NheI)

FP (-3832):

GCT AGC TGG AGA CAT AGA TAA GGG GCA TTG TG (NheI)

FP (-4074):

GCT AGC TTT TCT ATA ACC CAA TGT CTC TTG GAT GC (NheI)

mGr construct (NM_008173.3)

FP:

GCT AGC ATG GAC TCC AAA GAA TCC TTA GCT C (NheI)

RP:

GGA TCC TCA TTT CTG ATG AAA CAG AAG CTT TTT G (BamHI)

- **Site-directed mutagenesis oligonucleotides**

PEPT1

-390 mut +:
CAG CTG CCA ACC GTC ACA CCG TGT TTT GCC TCC CCA CCC

-390 mut -:
CAC GGT GTG ACG GTT GGC AGC TGT GTG TGT GTG TGT GTG TGT G

1500 mut +:
CAT ATT TTT GGC CTA CTT TTT GAT GGG GTT GTT TGA TTT TTT TC

1500 mut -:
CCA TCA AAA AGT AGG CCA AAA ATA TGA ACA GAC CCT TCT CAA AAG

r*Pept1*

1495 mut +:
CCT CAA CAT CAC GAA TTG CAC CCT ACC GAG GAA ACC AAC CAA AGG

1495 mut -:
CGG TAG GGT GCA ATT CGT GAT GTT GAG GTC CAA AAC TTC GAC ATC

- **EMSA oligonucleotides**

PEPT1 PPREs

-389/-401:
AGC TAC GGT GGG ACA GTG GTC AGC TGT

-1500/-1512:
AGC TAA AAG TGG GCA AAG GAT ATG AAC

m*Pept1* PPREs

-1283/-1295:
AGC TTC CTT TTA CCC TTG AAC TGT TGC

-1762/-1774:
AGC TGC ATG TGT TCT GTG TCC TGC AGG

-1828/-1840:
AGC TGT CTA TGT CCT CTA CAC TGA GAG

-1862/-1874:

AGC TGG GCC TCT TCT TTG ACC TCA CTC

r*Pept1* PPRE

-1475/-1487:
AGC TTA GGG TGA ACT TTG TGC TGT TGA

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